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(54) Title: PREPARATION AND USE OF POLYANIONIC POLYMER-BASED CONJUGATES TARGETED TO VASCULAR ENDOTHELIAL CELLS (57) Abstract <p>This invention discloses new targeted conjugates for the delivery of a compound, and particularly, a steroid, to vascular endothelial cells. The conjugates comprise two components, preferably linked by a selectively-hydrolyzable bond, such as an acid-labile bond or enzyme-sensitive bond. The first component, a polyanionic polymer, and preferably, a polysulphated polymer such as a heparin-derivative, specifically directs the conjugate to vascular endothelial cells. The second component is a selected agent, such as a steroid, which exerts a specific effect on the target cell following its release. In particular, the present invention provides novel conjugated angiogenesis inhibitors, for use in the treatment of pathogenic conditions including cancer, arthritis, and diabetic blindness. An inhibitor comprising a heparin derivative and the anti-angiogenic steroid, cortisol, is herein shown to be markedly acid-labile, to suppress DNA synthesis and cell migration in human umbilical vein endothelial cells, to retard or abolish (depending on the route of injection) the vascularization of sponges <i>in vivo</i> and to retard lung tumor growth in mice by 65 %. No adverse effects of the conjugate were detected, and equivalent treatments with a mixture of heparin plus cortisol were significantly less effective in all cases.</p>		

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DESCRIPTIONPREPARATION AND USE OF POLYANIONIC POLYMER-BASED
CONJUGATES TARGETED TO VASCULAR ENDOTHELIAL CELLS

5

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of
10 co-pending application U.S. Serial No. 07/856,018, filed
March 23, 1992. The government owns certain rights in
the present invention pursuant to NIH grant number CA-
54168.

15 1. Field of the Invention

The present invention provides novel conjugates for
use in targeting a selected agent, and particularly, a
steroid, to vascular endothelial cells. These conjugates
20 comprise two components preferably linked by a
selectively-hydrolyzable bond, such as an acid-labile
bond. Firstly, a polyanionic compound such as a
polyanionic polymer, which directs the conjugate to
vascular endothelial cells, and secondly, a selected
25 agent, such as a steroid, which exerts its action
following cellular release. The invention provides novel
conjugates that function as targeted angiogenesis
inhibitors that are proposed for use in the treatment of
pathological conditions such as cancer, arthritis, and
30 diabetic blindness. Preferred inhibitors are those in
which the polyanionic compound is a polysulfated polymer
such as a heparin derivative, conjugated to a steroid
with anti-angiogenic activity, such as cortisol, or
derivatives and variants thereof.

35

2. Description of the Related Art

The control of endothelial cell proliferation is a vital part of normal homeostatic mechanisms. A
5 disturbance in this process can result in, for example, excessive or inappropriate endothelial cell proliferation or activation which is often associated with disease processes. For example, the proliferation of vascular
10 endothelial cells is vital to angiogenesis, the formation of new blood vessels, which in turn, is associated with many disabling or life-threatening disorders. These include cancer (Algire et al., 1945; Tannock, 1968; Folkman, 1972) and other pathological conditions such as
15 diabetic retinopathy, atherosclerosis, rheumatoid arthritis, synovitis, psoriasis, dermatitis, endometriosis, encephalitis and tonsillitis (Brown & Weiss, 1988, Waltman et al., 1978; Gartner & Henkind, 1978; Moses & Langer, 1991). It is known that
20 angiogenesis rarely occurs in healthy adult humans except during wound healing and during phases of the female reproductive cycle (Hobson & Denekamp, 1984).

In solid tumors, vascular endothelial cells divide about 35 times more rapidly than those in normal tissues,
25 except the uterine epithelium (Denenkamp & Hobson, 1982). Such inappropriate proliferation is necessary for tumor growth and metastasis (Folkman, 1986). Vascular endothelial cell growth and division is also important in chronic inflammatory diseases such as rheumatoid
30 arthritis, psoriasis and synovitis, where these cells proliferate in response to growth factors released within the inflammatory site (Brown & Weiss, 1988). In atherosclerosis, formation of the atherosclerotic plaque is triggered by a monoclonal expansion of endothelial
35 cells in blood vessels (Alpern-Elran et al., 1989). Furthermore, in diabetic retinopathy, blindness is thought to be caused by basement membrane changes in the

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eye, which stimulate uncontrolled angiogenesis and consumption of the retina (West & Kumar, 1988).

Endothelial cells are also involved in graft rejection. In allograft rejection episodes, endothelial cells express proadhesive determinants that direct leukocyte traffic to the site of the graft. It is believed that the induction of leukocyte adhesion molecules on the endothelial cells in the graft may be induced by locally-released cytokines, as is known to occur in an inflammatory lesion.

As endothelial cells are involved in a wide variety of processes, it has been reasoned that drugs targeted to such cells may be of wide-ranging use clinically. Perhaps most importantly, angiogenesis inhibitors could potentially be of use in the treatment of those diseases, mentioned above, whose pathogenesis is influenced or maintained by the proliferation of vascular endothelial cells. Angiogenesis inhibitors would be particularly advantageous in the treatment of cancer, in which one of the current major problems is the emergence of drug-resistant malignant cells.

Within the last decade, several inhibitors of angiogenesis have been identified (Langer et al., 1976; Taylor & Folkman, 1982; Sharpe et al., 1990, Good et al., 1990, Ingber et al., 1990). For example, anti-angiogenic therapy, in the form of oral or subcutaneous administration of heparin and the steroid cortisone, has been reported to have an anti-tumor effect in mice bearing established tumors of various types (Folkman et al., 1983). It was believed that the heparin, or heparin metabolites, acted together with the cortisone to inhibit angiogenesis and tumor growth. Unfortunately, the magnitude of the anti-tumor effect varied with the batch of heparin used, and later studies from several

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laboratories have subsequently reported no (Ziche et al., 1985; Penhaligon & Campejohn, 1985), or only modest (Sakamoto et al., 1986; Benrezzak, 1989), anti-tumor effects.

5

The mechanism underlying the additive anti-angiogenic activity of heparin and cortisone has not been positively identified, although it may be related to the fact that the mixture increases the rate of dissolution of the basement membrane beneath newly formed capillaries (Ingber et al., 1986). Structure-activity studies have shown that steroids lacking glucocorticoid and mineralocorticoid activity can act in conjunction with heparin to inhibit angiogenesis. These studies have led to the description of a new biological activity of steroids, called the angiostatic activity (Crum et al., 1985).

More recently, the synthetic heparin substitute, β -cyclodextrin tetradecasulfate, was reported to augment the anti-angiogenic effects of angiostatic steroids on corneal neovascularization in rabbits when applied locally or topically (Folkman et al., 1989). It was suggested that the β -cyclodextrin tetradecasulfate might act by forming a non-covalent complex with the steroid and promote its binding to the surface of endothelial cells.

Other potential uses of modulating endothelial cell activity include the treatment of inflammatory responses or the stimulation of growth and proliferation. In the latter instance, this would prove useful in stimulating blood vessel growth, for example, in wound repair after accidental injury or surgery, or in promoting the healing of gastrointestinal lesions such as ulcers. Again, various steroids have potential for use in this area.

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The clinical use of many agents, including steroids, whether designed to be inhibitory or stimulatory, is often limited by their side effects and toxicities. For example, steroids are known to have negative effects on bone and lymphoid tissues, which can reduce their overall dosages and their therapeutic effectiveness. In particular, steroid treatment is known to be associated with osteoporosis and immunosuppression. Naturally, these are serious drawbacks which currently limit the use of steroids in the treatment of human disorders. Most toxicity can generally be attributed to a lack of selective action, i.e. it is due to the tendency of the drug to exert its effects on cells indiscriminately.

Accordingly, there is currently a substantial need for an improved means of treating diseases or processes involving endothelial cells, and particularly, processes involving inappropriate endothelial cell growth or proliferation. Furthermore, there is a particular need for this means of treatment to have increased selectivity and specificity, allowing the advantageous use of various agents whilst limiting any toxicity of the agents on tissues other than the endothelial cell tissue.

25

SUMMARY OF THE INVENTION

The present invention seeks to overcome these and other drawbacks inherent in the prior art by providing novel endothelial cell-targeted conjugates for the delivery of a selected agent, such as a steroid, to vascular endothelial cells. The targeting component of the conjugate is a polyanionic compound, such as a polyanionic polymer, and preferably a polysulfated polymer such as a heparin derivative, which is preferably linked to the selected agent by a selectively-hydrolyzable bond, for example, an acid-labile bond. The

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selected agent, preferably a steroid, exerts its action on the target cells following release. In certain aspects, the present invention provides conjugates that function as targeted inhibitors of angiogenesis, for use
5 in the treatment of pathological conditions such as cancer, arthritis, and diabetic retinopathy. Particularly preferred inhibitors are those in which a heparin derivative is conjugated to a steroid with anti-angiogenic activity, such as cortisol, and derivatives
10 and variants thereof.

A key aspect of the present invention is the creation of an "endothelial cell-targeted conjugate". The term "endothelial cell-targeted conjugate", as used
15 herein, indicates that the conjugate selectively binds to vascular endothelial cells as opposed to other cell types except the cells of the reticuloendothelial system. "Targeting" of a compound in this manner is intended to refer to, not only the process by which the compound
20 recognizes and binds to specific components of vascular endothelial cells, but also to its uptake by such cells.

The "targeting component" of these novel conjugates is the component that directs the conjugate to its
25 intended site of action, i.e. to vascular endothelial cells. The targeting component therefore controls the biodistribution of the conjugate, and as such, may be considered as a delivery vehicle for the selected agent of the conjugate. Targeting components of the present
30 invention are defined in a general sense as polyanionic compounds, and more particularly, as polysulphated or polysulphonated compounds.

The polyanionic compounds of the present invention
35 may either be polymers themselves or may be smaller molecules such as monomers and dimers which are nevertheless polyanionic. "Polyanionic" refers to a

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compound which has more than two anionic groups, and preferably, to a compound which has a larger number of such groups, such as, for example, in the order of about 4, 5 or 6 anionic groups up to about 9 or 10 anionic groups or more. Of course, where the polyanionic compound is also a polymer, it may have a very large number of anionic moieties. Polyanionic non-polymeric compounds which are considered to be particularly useful are suramin and suramin derivatives and analogues. Other examples of such compounds include trypan blue and Evans blue dye. As smaller molecules are less immunogenic than polymers, certain advantages may result from using a polyanionic small molecule rather than a polymer as the targeting agent in embodiments concerning the administration of a conjugate to an animal.

Suramin is an angiostatic agent which has been used in the treatment and prophylaxis of trypanosomiasis caused by *T. rhodesiense*, *T. hippicum* and *T. gambiense*, and for the treatment of onchocerciasis and pemphigus. It has moderate antiviral activity, and has even been employed as an AIDS therapeutic and as an anti-cancer agent. Suramin can be prepared synthetically, as disclosed herein, or obtained from the CDC Drug Service, Centers for Disease Control, Atlanta GA 30333.

It is contemplated that both synthetic as well as naturally occurring polyanionic compounds and polymers will be useful in the present invention. Of course, where administration of the conjugate to a human is contemplated, one will desire to ensure that the compound or polymer employed is biocompatible, i.e. that it does not cause any substantial adverse effects when administered to a human subject. Virtually all naturally occurring polysulfated polyanions will be suitable in this regard.

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Exemplary polymers useful in accordance with this invention will include both *N*- and *O*-sulfated polyanionic polysaccharides. Of these, the polysaccharides having free carboxyl groups are considered to be particularly useful because the carboxyl group can serve as a convenient attachment point for drugs and intermediate spacer groups. Such carboxyl-containing sulfated polysaccharides include heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, sulfated alginic acid and the like. However, a wide variety of naturally-occurring and synthetic *N*- and *O*-sulfated polyanionic polysaccharides lacking carboxyl groups could also be used in accordance herewith. These include polymers such as keratan sulfate, dextran sulfate, sulfated cyclodextrins, sulfated chitin, sulfated chitosan, pentosan polysulfate and the like. Each of the foregoing, whether *N*- or *O*-sulfated, can be derivatized to introduce functional groups that can serve as attachment points for drugs, other selected agents, or intermediate spacer groups.

In addition to the polysaccharide-based polymers such as those described above, it is contemplated that a variety of synthetic organic polymers will also prove to be employable in the creation of a targeted conjugate. For example, it is contemplated that polysulfated or polysulfonated polymers such as polystyrene sulfonate, sulfated polyvinyl alcohol, polyvinyl sulfate or even polyethylene sulfonate could be employed in place of the more preferred polysaccharides and nonetheless obtain a useful conjugate in accordance with the present invention. As with the polysaccharides, it will generally be preferred to employ polyanionic polymers wherein the negatively charged moieties are sulfate or sulfonate groups. However, it is proposed that other negatively charged groups may be incorporated onto the polymer backbone, such as phosphate, or even carboxyl

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groups, and that such groups will suffice to achieve some degree of selective targeting.

As mentioned above, in certain preferred
5 embodiments, the inventor contemplates the use of heparin
as the targeting component of a given conjugate because
it is readily available, relatively inexpensive, its
chemistry and medicinal properties have been extensively
studied. In preferred embodiments, it is contemplated
10 that a non-anticoagulating derivative of heparin will be
used. One of the physiological roles of heparin is to
act as an anticoagulant, i.e. to inhibit the clotting
reactions of the blood. A non-anticoagulating derivative
is therefore a compound devoid or virtually devoid of
15 this activity. The absence of anti-coagulant activity
from the conjugate is considered to be advantageous as it
will not perturb the physiological reactions occurring in
the recipient, and hemorrhage is unlikely to be a problem
with heparin-steroid treatment.

20

Heparin can be converted to a virtually non-
anticoagulating form during the novel conjugation
procedure described herein. The anti-coagulating
activity of heparin is known to be associated in part
25 with the carboxyl groups of the molecule, or more
exactly, with the carboxyl groups of the glucuronic or
iduronic residues within the heparin structure. These
are precisely the groups that are modified on the initial
derivatization of heparin to obtain a heparin hydrazide.
30 Therefore, the modification of some or all of the heparin
carboxyl groups is advantageous in two respects: it
provides the platform for further condensation reactions
to create a heparin conjugate, and it renders the heparin
derivative virtually devoid of anti-coagulating activity.

35

It is contemplated that heparin conjugates prepared
in accordance with preferred embodiments of the present

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invention will have less than 2.5%, and preferably, less than 1% of the anticoagulating activity of native (unmodified) heparin. A standard method for use in measuring the anticoagulant activity of a compound is to
5 determine its ability to inhibit the coagulation of human plasma induced by Russell's viper venom. To conduct an assay of this kind, one would prepare a solution of the test compound in a suitable buffer, such as phosphate-buffered saline (PBS), and mix with fresh citrated human
10 plasma at 37°C in glass tubes. After a short period of time, such as 1 minute, one would add Russell's viper venom (e.g., Sigma# RVCL-1) to the tube and mix. After a short period of time, such as 15 seconds, prewarmed CaCl_2 solution should be added, the contents mixed, incubated
15 at 37°C, and the time taken for the plasma to clot determined. To calculate the % anticoagulant activity of a heparin derivative or conjugate, one would compare the clotting time for plasma treated with that compound to the clotting time for plasma treated with native
20 (unmodified) heparin which would, by definition, be 100%.

The sulfate groups of the heparin molecule have been determined to be important for the tight binding of heparin derivatives to vascular endothelial cells.
25 Therefore, a further aspect of this invention is to create a heparin conjugate in which the heparin derivative has one or more unmodified sulfate residues, i.e. a heparin derivative in which one or more of the naturally-occurring sulfate residues remain unchanged
30 during the preparation of the conjugate. The conjugation procedure described herein is conducted so that the sulfate residues remain unaltered and can direct the binding of the conjugate to the sulfated polyanion receptors.

35

It is not believed to be necessary to routinely determine the ability of a targeted conjugate to bind to

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vascular endothelial cells during its construction, so long as one does not radically modify the sulfate groups of the molecule. However, if desired, one could examine this property, for example, by conducting a binding
5 assay. To conveniently perform such an assay, one would label the conjugate, for example with a radiolabel, and determine its ability to bind to vascular endothelial cells in culture, such as human umbilical vein
10 endothelial (HUVE) cells. This would be achieved by incubating the cells with the conjugate in a medium to allow binding, removing any unbound conjugate by washing, and then measuring the cell-associated label, i.e. the cell-associated radioactivity. One could compare the
15 binding of the test conjugate with that of native targeting molecule, for example, heparin.

Various selected agents are proposed to be of use in accordance herewith. The term "selected agent" is used herein simply to refer to the component of the conjugate,
20 other than the targeting component, which is delivered to the endothelial cells by the targeting component. Possible selected agents include radioisotopes, drugs which inhibit or promote DNA synthesis, drugs which suppress or enhance gene transcription, drugs which
25 affect cell surface functions and, indeed, any agent that one may wish to target to an endothelial cell. Pharmacological agents considered to be of particular use include those which, for example, are known to either stimulate or inhibit cellular proliferation.
30 Pharmacological agents of this kind include ionophores, antimetabolites, autacoids, vinca alkaloids, epipodophyllotoxins, alkylating agents, antibiotic chemotherapeutic agents (see Table I), and in preferred
embodiments, steroids, which are considered to be
35 particularly useful as selected agents in conjugates of the present invention.

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In important embodiments, the present invention provides conjugates that function as inhibitors of angiogenesis, i.e. that act to inhibit the growth of blood vessels. In such conjugates, one would naturally
5 choose to use a pharmacological agent with anti-angiogenic activity, such as a steroid. It is contemplated that suitable steroids for use in these embodiments include those in Table II and derivatives thereof. In certain embodiments, the steroid cortisol is
10 preferred because it is inexpensive, is straightforward to conjugate to heparin derivatives, and, when thus conjugated, efficiently inhibits angiogenesis.

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TABLE I
CHEMOTHERAPEUTIC AGENTS

Class	Type of Agent	Nonproprietary Names (Other Names)
Alkylating Agents	Nitrogen Mustards	Mechlorethamine (HN ₂)
		Cyclophosphamide Ifosfamide
		Melphalan (L-sarcolysin)
		Chlorambucil
	Ethylenimines and Methylmelamines	Hexamethylmelamine
		Thiotepa
	Alkyl Sulfonates	Busulfan
	Nitrosoureas	Carmustine (BCNU)
		Lomustine (CCNU)
		Semustine (methyl-CCNU)
		Streptozocin (streptozotocin)
	Triazenes	Decarbazine (DTIC; dimethyltriazenoimidazolecarboxamide)
Antimetabolites	Folic Acid Analogs	Methotrexate (amethopterin)
	Pyrimidine Analogs	Fluorouracil (5-fluorouracil; 5-FU) Floxuridine (fluorodeoxyuridine; FUDR)
		Cytarabine (cytosine arabinoside)

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	Class	Type of Agent	Nonproprietary Names (Other Names)
5	Antimetabolites continued	Purine Analogs and Related Inhibitors	Mercaptopurine (6-mercaptopurine; 6-MP) Thioguanine (6-thioguanine; TG) Pentostatin (2'-deoxycoformycin)
10	Natural Products	Vinca Alkaloids	Vinblastine (VLB) Vincristine
		Epipodophyllotoxins	Etoposide Teniposide
15		Antibiotics	Dactinomycin (actinomycin D) Daunorubicin (daunomycin; rubidomycin) Doxorubicin Bleomycin Plicamycin (mithramycin) Mitomycin (mitomycin C)
		Enzymes	L-Asparaginase
		Biological Response Modifiers	Interferon alfa

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5

Class	Type of Agent	Nonproprietary Names (Other Names)
Miscellaneous Agents	Platinum Coordination Complexes	Cisplatin (<i>cis</i> -DDP) Carboplatin
	Anthracenedione	Mitoxantrone
	Substituted Urea	Hydroxyurea
	Methyl Hydrazine Derivative	Procarbazine (N-methylhydrazine, MIH)
	Adrenocortical Suppressant	Mitotane (<i>o,p'</i> -DDD)
		Aminogluthethimide

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TABLE II
STEROIDS* FOR USE IN TARGETED ANGIOGENESIS INHIBITORS

	Tetrahydrocorticosterone	Tetrahydrocortexolone
	Cortisol (hydrocortisone)	Prednisone
5	Tetrahydrocortisol	Triamcinolone
	11 α -epihydrocortisol	Alclometasone
	Cortisone	Amcinonide
	Tetrahydrocortisone	Clobetasol
	Corticosterone	Clobetasone
10	Deoxycorticosterone	Clocortolone
	Cortexolone	Desonide
	Beclomethasone dipropionate	Desoximetasone
	Betamethasone	Diflorasone
15	Dexamethasone	Fluocinolone acetonide
	Flunisolide	Fluocinonide
	Methylprednisolone	Fluorometholone
	17 α -hydroxyprogesterone	Fluocortolone
	Tetrahydro S	Flurandrenolide
20	Paramethasone	Halcinonide
	Prednisolone	Medrysone
	Pregnenolone	Mometasone

*Plus derivatives thereof, such as the 21-phosphate, 21-hemisuccinate, 21-valerate, 21-sulfate derivatives.

The term anti-angiogenic (or angiostatic) steroid arose following studies on steroids lacking significant glucocorticoid and mineralocorticoid activity, such as tetrahydro S, that inhibited angiogenesis under certain conditions. Although useful, it will be appreciated that the creation of anti-angiogenic conjugates in accordance with the present invention is not limited to the use of steroids which are known to lack significant

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glucocorticoid and/or mineralocorticoid activity. Accordingly, any steroid with proven, or even possible, anti-angiogenic activity may be coupled to heparin to form a conjugate, the anti-angiogenic properties of which can then be examined. Furthermore, as the conjugates are specifically targeted to vascular epithelia, it is contemplated various steroids which have previously been associated with adverse clinical effects may now be used advantageously in the inhibition of angiogenesis.

In other embodiments, the present invention contemplates targeted conjugates that function as promoters of angiogenesis for use in stimulating blood vessel growth. Examples of selected agents or drugs that might be used in this way include phorbol esters, adenosine, inosine, hypoxanthine, nicotinamide, prostaglandin E2, and calcium ionophores such as nimodipine. It is envisioned that such conjugates would be of use in the treatment of conditions and diseases wherein a limited blood supply poses a problem, for example in the treatment of placental insufficiency, or the healing of gastric ulcers, or in the stimulation of wound repair.

The inventor contemplates that the components of the novel conjugates may be linked by a disulfide or trisulfide bond thus: polyanionic compound-S-S-(S)-selected agent, for example, heparin-S-S-S-calicheamicin. However, in preferred embodiments, the bond between the targeting component and the selected agent of the conjugates will be a selectively-hydrolyzable bond. The term "selectively-hydrolyzable bond", as used herein, refers to a bond which is stable unless exposed to particular conditions, i.e. unless placed within a certain environment or in contact with a particular agent. Such bonds include, for

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example, acid-labile and enzyme-sensitive bonds. In these cases, the "environment" or "agent" capable of inducing hydrolysis is an acidic pH, and a specific enzyme, respectively. Such selective-release design features allow the conjugates to remain intact whilst in the blood stream or other extracellular environments, and to be hydrolyzed only following binding to, or uptake into, vascular endothelial cells.

10 In certain embodiments, the selectively-hydrolyzable bond of the conjugate will be an acid-labile bond. As used herein, the term "acid-labile" is intended to refer to a conjugate (or the bond joining the two components of the conjugate) that is hydrolyzed simply by exposure to an acidic environment, and particularly, by exposure to an environment with a pH of 4.8 or lower. Conjugates with a half life of between 10 minutes and 24 hours, and preferably of between 15 minutes and 2 hours, in dilute solutions of 50 mM or less at 20°C and pH 4.8 are particularly preferred. An accompanying property of such conjugates is their stability at near-neutral pHs. The term "stable at near-neutral pHs" is herein intended to refer to a conjugate that is not substantially hydrolysed at pHs in the range of pH 7.4 to pH 7.6. For example, conjugates having less than 50%, and preferably, less than 10% breakdown after 24 hours in dilute solutions of 50 mM or less at 20°C and pH 7.4 are also particularly preferred.

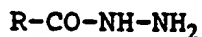
30 A useful method for analyzing the pH stability of a conjugate is to examine its UV spectrum over a pH range. The dissociation of the conjugate is determined by measuring the appearance of free cortisol, which is known to have a maximum at 242 nm. Firstly, the UV spectrum (from 400nm to 200nm) of the conjugate in distilled water may be scanned, then the pH of the conjugate solution made more acidic and the UV spectrum repeatedly re-

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scanned, whilst keeping the temperature constant. The half-life of the decomposition can be calculated as the time for the OD at 274 nm to decay to half its initial value. The % breakdown of the conjugate at a given time
5 can be calculated as the % of free cortisol which has formed over the time period, with time 0 taken as 0% breakdown.

Typical acid-labile linkages believed to be useful
10 in connection with the present invention include those that employ a Schiff's base linkage, for example, linkages incorporating the condensation product of an aldehyde or ketone with a hydrazine, a hydrazide, a primary or secondary amine or their derivatives. A
15 particularly preferred Schiff's base linkage for use in accordance herewith is an acyl hydrazone bond, formed when a hydrazide derivative of heparin, or other polyanionic compound or polymer, is condensed with a selected agent containing a ketone or aldehyde group. It
20 is proposed that the heparin or other polyanionic compound or polymer can be derivatised to introduce hydrazide-terminating side chains of various lengths. The simplest hydrazide-terminating side chain contemplated has the structure:

25



where R = polyanionic compound or polymer

30

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Longer hydrazide-terminating side chains are also proposed to be of use, for example, linkers of the formula:



wherein $n = 1-20$ and

R = polyanionic compound or polymer

10

In preferred embodiments, the inventor contemplates the use of hydrazide linkers that contain from 1 to 4 central CH_2 groups, i.e. from $n = 1$ to $n = 4$.

15

Other possible acid-labile linkages that would be suitable incorporate ortho ester, acetal and ketal functionalities that undergo acid-catalyzed dissociation but are base-stable; and cis-aconitic, maleic, or citraconic acid functionalities having a free carboxyl group juxtaposed to the acid-sensitive amide or ester linkage.

20

In further embodiments, the selectively-hydrolyzable bond of the conjugate will be an enzyme-sensitive bond.

25 The term "enzyme-sensitive", as used herein, is intended to refer to a conjugate (or the bond joining the two components of the conjugate) that is hydrolyzed by exposure to a specific enzyme. Examples of such bonds include esters, peptides, amides, phosphodiester, and

30 even glycosidic bonds, which are hydrolysed by exposure to an esterase, protease or peptidase, amidase, phosphodiesterase and glycosidase, respectively. Of course, in designing an enzyme-sensitive bond, one will generally desire to avoid such bonds that could be

35 hydrolysed by exposure to components normally present in the blood stream, such as certain esters.

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It is contemplated that the presence of an enzyme-sensitive bond within a conjugate of the present invention may add a further dimension to the utility of the conjugate. In this manner, conjugates could be engineered that are hydrolysed only by exposure to an enzyme known to have a precise cellular location, including enzymes associated with the plasma membrane, the endocytotic vesicles and the lysosomes.

Various side chains of a single polyanionic targeting molecule, such as heparin, can be modified by the addition of a selected agent to create a conjugate in accordance with the present invention. The molar ratios of selected agent to targeting component in a given conjugate can therefore lie between 0.1:1 and 80:1, depending on the particular polyanionic compound or polymer and selected agent utilized. In embodiments concerning the conjugation of steroids, molar ratios of greater than one, and preferably, of greater than 5:1 are contemplated.

The present invention further provides a method for preparing an endothelial cell-targeted polyanionic compound or polymer conjugate comprising the following steps:

(a) condensing in a reaction mixture the selected polyanionic compound or polymer with a derivatizing agent to form a derivatized polyanionic compound or polymer;

(b) removing from the reaction mixture any unreacted material from said derivatised polyanionic compound or polymer;

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(c) adding to the reaction mixture a molar excess of the selected agent to be conjugated; and

5 (d) incubating the reaction mixture to provide a polyanionic compound or polymer-agent conjugate.

10 In certain embodiments, the polyanionic compound used to form the targeting component of the conjugate will be a polymer, preferably heparin, and even more preferably, it will high molecular weight heparin, e.g. with a molecular weight on the order of 5,000 to 25,000. In other embodiments, a smaller polyanionic molecule such
15 as suramin may be employed. Particularly preferred derivatizing agents for use in accordance with the above method are hydrazine and hydrazide derivatives. In using such agents for the preparation of a heparin conjugate, the initial step in the procedure is the creation of a
20 heparin hydrazide, using an excess of water soluble carbodiimide, such as EDC, at an acidic pH.

In the preferred mode of synthesis of a conjugate between, for example, heparin and an agent having an
25 available ketone, or aldehyde group, such as a steroid, the initial derivatization can be achieved by carrying out the reaction in a similar manner to that described for the synthesis of heparinylglycine (Danishefsky & Siskovic, 1971). To condense heparin with adipic
30 dihydrazide one would prepare a sample of high molecular weight heparin at an appropriate concentration, such as 10mg/ml, and add adipic dihydrazide to a final concentration of approximately 0.5M. The pH of the solution should then be adjusted to, for example, about
35 pH 4.75 by the addition of a suitable acid, such as 1M HCl. Next, one would add a freshly prepared solution of EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) in

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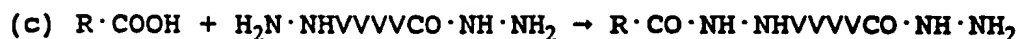
water was added to give a final EDC concentration of approximately 10.7 mg/ml, and allow the reaction to proceed at room temperature while maintaining the pH at approximately 4.75 by the periodic addition of, for example, 1M HCl. Following pH stabilization (~2-3 hours), the mixture should be kept at 4°C for a longer period of time, such as 48 hours, and then dialyzed extensively into 1mM HCl, for example, using four changes of dialyzate over 4 days. One would then dialyze the mixture into a suitable buffer, such as 0.1M sodium acetate, pH 4.0 and adjust to an appropriate concentration, such as 1mg/ml.

However, the inventor contemplates that other methods of derivatizing a polyanionic compound or polymer, such as heparin, to permit the subsequent attachment of a selected agent having a free aldehyde or ketone group will also be suitable. For example, the initial derivatization can take advantage of available -COOH groups, such as those of the iduronic or glucuronic acid residues of heparin, as in the following condensation reactions which might, for example, employ a carbodiimide as the condensing agent:



where VVVV can be any aliphatic or aromatic intermediate grouping, such as $(CH_2)_n$, $(CH_2O)_n$, $(CH_2 \cdot CO \cdot NH)_n$, $(CH(OH))_n$ and derivatives thereof, also wherein $n = 0-20$ or so, with $n = 0-8$ being preferred, and $n = 0-4$ being even more preferred.

35



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5 Furthermore, derivatization reactions involving -
 NH·CO·CH₃, are also contemplated to be of use in
 accordance with the present invention. Such groups can
 be found on the sugar residues of polyanionic compounds
 and polymers, such as the *N*-acetylglucosamine groups of
 10 heparin. In performing a derivatization reaction of this
 kind, one would first partially or completely deacetylate
 such groups, for example, using an acid- or base-
 catalysed reaction, or by hydrazinolysis in aqueous
 hydrazine containing 1% hydrazine sulfate (as represented
 15 below).



20 Selected agents containing, for example, a ketone or
 aldehyde, a carboxyl group, a sulfonic acid group or
 derivatives thereof such as carboxylic anhydrides or
 sulfonyl halides could then be reacted with the amine
 groups of the polyanionic compound or polymer.

25 Where compounds or polyanionic polymers containing
N-sulfated sugars are used as a targeting component,
 these groups can also be derivatized. Firstly, one would
 partially or completely de-*N*-sulfate, for example, using
 30 dimethylsulfoxide containing 5% H₂O or MeOH at 20°C - 50°C
 (as represented below).

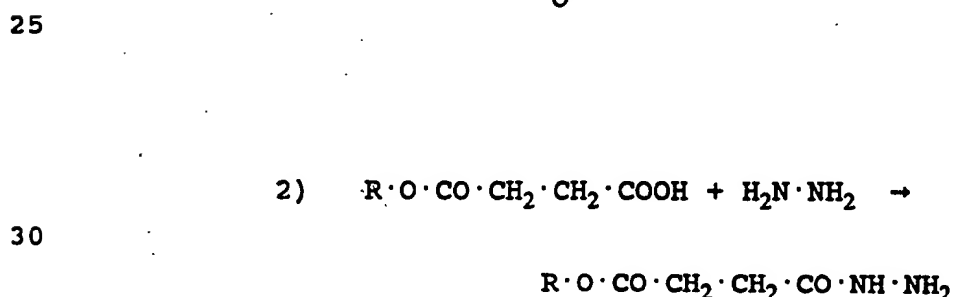
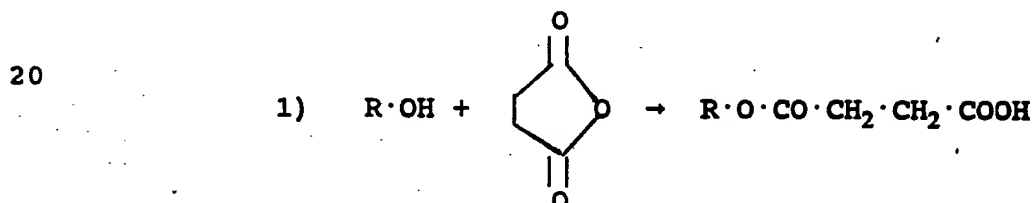


35 Selected agents containing, for example, a ketone or
 aldehyde, a carboxyl group, a sulfonic acid group or
 derivatives thereof such as carboxylic anhydrides or

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sulfonyl halides could then be reacted with the amine groups of the polyanionic compound or polymer.

Further derivatizing reactions involving sugars of polyanionic compounds or polymers are also contemplated, for example, those utilizing the OH groups of the sugar residues. As represented below, one method contemplates the use of an anhydride, for example, succinic anhydride, in the first step to introduce a carboxyl group. In the second step, any one of the compounds detailed above in examples (a) to (e) could be condensed with the carboxyl group, for example, hydrazine. Following derivatization according to these steps, the compound or polymer could again be reacted with selected agents containing, for example, an aldehyde or ketone, a carboxylic group, a sulfonic acid group or derivatives thereof such as carboxylic anhydrides or sulfonyl halides.



To create a covalently bonded heparin-agent conjugate, one would form a reaction mixture by adding a solution of an agent with one or more free aldehyde or ketone groups, such as a steroid, to the heparin-hydrazide to give an appropriate final concentration, for example, in the case of a steroid, of approximately 3 to 4 mg/ml steroid. Incubation of this reaction mixture at a temperature of, for example, between 15°C and 60°C,

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leads to the formation of the heparin-steroid conjugate product. A suitable period of time for this incubation is considered to be between approximately 1 hour and 14 days, depending on the reactivity of the steroid. After
5 formation, the product can be stabilized by adjusting the pH to ~pH 10 with a suitable alkali, such as 1M NaOH, and then dialyzing extensively into water whose pH has been adjusted to 10. For long-term storage, the dialyzed
product can be freeze-dried and placed in a desiccator at
10 4°C.

It is envisioned that endothelial cell-targeted conjugates prepared in accordance with the present invention may be advantageously used in a number of
15 different embodiments, both *in vivo* and *in vitro*. Importantly, such novel conjugates will have utility in the clinical treatment of a spectrum of human disorders. In its broadest sense, the present invention provides a universally applicable means of targeting any drug or
20 other selected agent to a vascular endothelial cell. Various conjugates can be thus synthesized, using different targeting polymers and selected agents, for use in a wide variety of treatment regimens. A particularly important aspect of the present invention is the use of
25 targeted conjugates as inhibitors of angiogenesis in the treatment of various conditions including cancer, arthritis, and diabetic retinopathy.

In certain embodiments, the present invention
30 concerns a method for introducing a steroid to a vascular endothelial cell. To accomplish this, one would simply prepare an endothelial cell-targeted steroid conjugate, as described above, and allow vascular endothelial cells to be exposed to the conjugate. Related methodological
35 embodiments provide a means for either stimulating or inhibiting DNA synthesis and cellular proliferation in vascular endothelial cells. To achieve these ends, one

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would again prepare a targeted steroid conjugate, as described above, and expose vascular endothelial cells to the conjugate. Naturally, to stimulate DNA synthesis one would employ a conjugate with an anabolic steroid, and to
5 inhibit DNA synthesis, an anti-angiogenic conjugate should be chosen.

In related methodological embodiments, the present invention provides a means for inhibiting the migration
10 of vascular endothelial cells. To achieve this, one would prepare an endothelial-cell targeted steroid conjugate using a steroid with anti-angiogenic activity, as described herein, and allow vascular endothelial cells to be exposed to the conjugate. The method for assaying
15 cellular migration preferred by the present inventor is the measurement of repair in cultured monolayers wounded by a scarring action. The degree of wound repair is determined in the presence and absence of an endothelial cell-targeted steroid conjugate, and the two values
20 compared. After wounding, the monolayer cultures are incubated at 37°C for a period sufficient to allow repair, such as 5 days, and their appearance, with and without the conjugate, is compared.

25 The methods described above are equally applicable to vascular endothelial cells *in vitro*, for example, in tissue culture, or *in vivo*. To achieve these effects in cultured endothelial cells, all that is required is to simply add the conjugate to the culture medium. Cultured
30 endothelial cells are readily available and include, for example, human umbilical vein endothelial (HUVE) and murine pulmonary capillary endothelial (MPCE) cells. To achieve like effects with endothelial cells in a living animal, one would introduce the targeted steroid
35 conjugate to the animal, where it would be specifically targeted to, and imported by, the vascular endothelial cells. Naturally, where the animal has a disease or a

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disorder, the administration of a conjugate provides an effective means of treatment. The direct effects of a conjugate on vascular endothelial cells, such as inhibition of DNA synthesis, are more difficult to
5 measure from experiments conducted *in vivo*. However, several assays are available to analyze the effects on the physiological processes occurring.

One assay which gives an indication of vascular
10 endothelial cell DNA synthesis, proliferation and migration is to examine the vascularization of implanted material, such as a sponge. To prepare an experimental animal (for example, a mouse) for a sponge
vascularization experiment, one would implant a sponge
15 disc with an internal cannula into an appropriate site, such as into a subcutaneous pouch, and exteriorize the cannula. To assess vascularization of the sponge, one would anaesthetize the animal, inject a sterile solution of a non-metabolizable labeled compound, such as ^{133}Xe in
20 saline, into the sponge via the cannula and then plug the cannula to prevent escape of the labeled compound. The radioactivity would then be measured, for example, using a collimated gamma scintillation detector situated 1cm above the sponge.

25

In an assay of this kind, freshly implanted sponges would be avascular and one would expect the ^{133}Xe to be cleared slowly. In contrast, in a fully vascularized sponge, the network of blood vessels would lead to rapid
30 clearance. Following injection of a targeted steroid conjugate into the experimental animal, or into the sponge via the cannula, one can thus determine its effect on the vascularization process by measuring the ^{133}Xe
clearance rate. In a study of this kind, one can also
35 subsequently dissect out the sponge and obtain transverse frozen sections for direct analysis. For example, one may wish to immuno-identify any blood vessels, and

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determine their numbers, appearance, and the presence of blood cells within them.

In particularly important embodiments, the present invention contemplates the clinical use of endothelial cell targeted steroid conjugates to treat several human diseases. Clearly, administering a conjugate of the present invention to an animal provides a means for either inhibiting, or stimulating, angiogenesis, i.e. for reducing or increasing the number of blood vessels and the blood supply to an area of the body. Inhibition of angiogenesis is an effective method by which to treat various diseases which are caused or perpetuated by an inappropriate blood vessel growth. Such disease include cancer, rheumatoid arthritis, diabetic retinopathy, atherosclerosis, synovitis, psoriasis, dermatitis, endometriosis encephalitis and tonsillitis. In the treatment of cancer, the administration of a heparin-steroid conjugate also provides a means to inhibit metastasis. Alternatively, the stimulation of angiogenesis would be useful in treating conditions such as placental insufficiency or gastric ulcers, or in the stimulation of blood vessel growth in wound repair. Conjugates of the present invention will also find utility in the control of inflammatory diseases.

The novel conjugates of the present invention may be administered alone or in combination with pharmaceutically acceptable carriers, in either single or multiple doses. Naturally, the pharmacological composition of a conjugate will be dependent on the route of administration chosen, which will in turn, be partly dependent on the condition being treated. The conjugates can be administered by one or more of a variety of routes including injection, topical administration, and release from a biodegradable drug-release capsule. Therefore, the conjugates, or pharmaceutical compositions

-30-

thereof, can be administered in a variety of dosage forms including, for example, injectable solutions, or encapsulated within a biodegradable polymer which acts as a slow-release capsule.

5

Suitable pharmaceutical carriers for use in conjunction with the present invention include inert solid diluents or fillers, sterile aqueous solution and various organic solvents, the choice of which will again vary according to the route of administration. For parenteral administration, including intravenous, intramuscular and subcutaneous injection, solutions of the conjugates in various oils, emulsions, or aqueous sterile buffers may be employed. The precise compositions and use of such pharmaceutical carriers will be known to those of skill in the art in light of the present disclosure.

20

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Structure of heparin-daunomycin and heparin-carminomycin conjugates.

25 Figure 2. Structure of heparin-mithramycin conjugates.

Figure 3. Predominant structure of directly-linked heparin-aminopterin conjugate.

30 Figure 4. Predominant structure of aminopterin-LALA-heparin conjugate.

Figure 5. Structure of heparin-adipic hydrazide-cortisol conjugate.

35

Figure 6. Dissociation of heparin-adipic hydrazide-cortisol at pH 4.8. The u.v. spectrum of cortisol

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coupled via C3 to heparin adipic hydrazide has a maximum at 274 nm, whereas free cortisol has a maximum at 242 nm. When heparin-cortisol (120 μ g/ml) is incubated at pH 4.8 it dissociates to give free cortisol with a half-life of 35 minutes at 20°C and 15 minutes at 37°C. The conjugate does not dissociate detectably at pH 7.4.

Figure 7. Dissociation of heparin-hydrazine hydrazide-cortisol phosphate (A) and heparin-adipic hydrazide-cortisol phosphate (B) at pH 4.8.

Figure 8. Effects of heparin steroid conjugates on DNA synthesis in cultured cells.

Figure 8A. Inhibition of DNA synthesis in HUVE cells by heparin-adipic hydrazide-cortisol. HUVE cells were allowed to adhere to gelatin-coated microplate wells for 24 hours and were then treated for 24 hours at 37°C with heparin-cortisol (O), with an equivalent mixture of unconjugated heparin hydrazide and cortisol (*), or with cortisol alone (Δ). Concentrations in the figure refer to the cortisol concentration in each treatment. The capacity of the cells to incorporate 3 H-Tdr into DNA was determined 24 hours later. 3 H-Tdr incorporation is expressed as a percentage of that in untreated control cultures.

Figure 8B. Inhibition of DNA synthesis in MPCE cells by heparin-adipic hydrazide-cortisol. MPCE cells were grown in 96 well plates for 24 hours and were then treated for 24 hours at 37°C with heparin-adipic hydrazide-cortisol conjugate (O), with an equivalent mixture of unconjugated HAH plus cortisol (*) or with cortisol (\square) or HAH (\blacksquare) alone. Concentrations on the lower axis refer to the cortisol concentration in each treatment and those on the upper axis refer to the heparin-adipic hydrazide concentration. The ability of the cells to incorporate

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^3H -Tdr into DNA was determined 24 hours later. ^3H -Tdr incorporation is expressed as a percentage of that in untreated control cultures (84,100 dpm for MPCE cells).

5 Figure 8C. Inhibition of DNA synthesis in MPCE cells by
heparin-adipic hydrazide-tetrahydro S. MPCE cells were
treated for 24 hours at 37°C with heparin-adipic
hydrazide-tetrahydro S (○), with an equivalent mixture of
unconjugated HAH plus tetrahydro S (●), or with
10 tetrahydro S (□) or HAH (■) alone. Concentrations on the
lower axis refer to the tetrahydro S concentration in
each treatment and those on the upper axis refer to the
heparin-adipic hydrazide concentration. The ability of
the cells to incorporate ^3H -Tdr into DNA was determined 24
15 hours later and is expressed as a percentage of that in
untreated control cultures (179,500 dpm).

Figure 9. Delayed vascularization of implanted sponges
in mice by administration of heparin-adipic hydrazide-
20 cortisol. Polyurethane sponges were implanted
subcutaneously into mice. The extent of vascularization
of the sponges was assessed at various times thereafter
by injecting ^{133}Xe into the sponges and measuring the rate
of disappearance of radioactivity. As vascularization
25 proceeds, the half-life of ^{133}Xe clearance falls from
about 25 minutes to about 7 minutes. In (a), starting 3
days after implantation, heparin-cortisol (0.78 mg) was
injected daily for 10 days directly into the sponge (○).
Other mice received equivalent quantities of a mixture of
30 unconjugated heparin hydrazide (0.65mg) plus cortisol
(0.13mg) (●), cortisol (0.13mg) alone (■), unmodified
heparin (0.33mg) plus cortisol (0.13mg) (▲), or diluent
(Δ). In (b), starting 3 days after implantation, mice
were injected intraperitoneally with 2.35mg heparin-
35 cortisol for 5 days and then with 1.17mg heparin-cortisol
for a further 7 days (○). Other mice received equivalent
quantities of unconjugated heparin hydrazide plus

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cortisol (•), or diluent (Δ). Each treatment group consisted of 10 mice.

Figure 10. Anti-tumor effects of heparin-adipic hydrazide-cortisol. Mice were injected subcutaneously with 3LL Lewis lung carcinoma cells. When the tumors reached approximately 0.3cm in diameter, the mice were injected daily with the heparin-cortisol conjugate (1mg) or with equivalent quantities of heparin hydrazide plus unconjugated cortisol or with diluent alone. Tumors were removed on day 10 after the onset of treatment and were weighed. The bars show the mean tumor weight per group \pm one S.D.

Figure 11. Possible Mechanism of Action of Heparin-Cortisol.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

20

Excessive or inappropriate endothelial cell proliferation or activation can contribute to the cause or maintenance of several disease processes. This includes angiogenesis, the formation of new blood vessels, which is associated with many disabling or life-threatening disorders in adults. Such pathological conditions include cancer, diabetic retinopathy, atherosclerosis, rheumatoid arthritis, synovitis, psoriasis, dermatitis, encephalitis and tonsillitis. Disturbed endothelial cell processes are also involved in inflammatory lesions and graft rejection, where the endothelial cells express proadhesive determinants that direct leukocyte traffic to the area.

35

As endothelial cells are involved in a wide variety of disorders, drugs targeted to such cells may be of wide-ranging use clinically. For example, angiogenesis

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inhibitors could be used in the treatment of those diseases, mentioned above, which are at least partially dependent on the aberrant proliferation of vascular endothelial cells. Such inhibitors would be particularly useful in the treatment of cancer, arthritis and diabetic retinopathy.

A variety of pharmacologic agents are known which exhibit anticellular, antiproliferative or anti-inflammatory actions, and could potentially be of use in the treatment of one or more conditions involving epithelial cell growth or activation. Unfortunately, many of these agents, particularly antiproliferative agents, exhibit profound side effects and toxicities which can limit their overall dosages or therapeutic regimens. Most toxicity can be attributed generally to a lack of selective action.

Many toxicities associated with anti-neoplastic therapy can be traced to the relatively non-selective mode or site of action of chemotherapeutic agents presently in use, which rely simply on an increased growth rate of most tumor cells as compared to most normal cells for their selectivity. Drugs such as Ara C, methotrexate, 5-FU, anthracyclines, mitomycin C, alkylating agents, as well as a variety of others, have toxicities attributable to the tendency of the drug to exert its anticellular effect on dividing cells indiscriminately. Similarly, while angiostatic steroids such as cortisol, hydrocortisone, dexamethasone, and the like, are attractive agents for potential use in anti-angiogenic therapy, they induce side effects such as osteoporosis and immunosuppression which reduce their clinical usefulness.

35

The targeting of drugs to specific sites within the body may be able to reduce their toxic effects on other

-35-

systems. One approach to the cell specific targeting of drugs has been the formation of antibody-drug conjugates, wherein the antibody directs the associated drug or toxin to a target cell such as a tumor having a cell surface antigen marker. A variety of tumor-directed antibody-drug conjugates have been prepared and tested for use as potential therapeutic agents, such as in the treatment of cancer. However, most drugs conjugated with antibodies, other than immunoconjugates of highly cytotoxic moieties such as ricin A chain molecules, have met with little success. The reason for this is unclear but is likely due to the inability to deliver a sufficient number of drug molecules to the target to be effective, or additionally it could be due to an inability to deliver the drug to the target in an active form, such as a inability to "release" the drug from its conjugate bound form.

Liposomal encapsulation constitutes another means of altering the pharmacologic properties of a drug, including altered biodistribution and reduced toxicity. Liposomal encapsulation involves the incorporation of molecules of the drug into a "capsule" of lipid materials, usually composed of uni- or multilamellar vesicles of various phospholipids. Unfortunately, the ability to form stable liposomes of a particular agent is somewhat limited, in that liposome stability is a function of a variety of parameters such as drug lipophilicity and other structural consideration. Thus, liposomal encapsulation has not proved to be applicable to a broad spectrum of agents. Furthermore, the ability of liposomal encapsulation to alter biodistribution is unpredictable at best. Thus, such encapsulation has not provided a particularly satisfactory broadly applicable means of targeting a selected drug in a uniform, tissue-specific manner.

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Accordingly, there has existed in the art a great need for an improved means to treat diseases or processes involving endothelial cells, and particularly, disorders involving endothelial cell growth or proliferation.

5 Because many drugs currently available for treating conditions of cell growth or activation have significant associated toxicities, and are not specific for selected tissues such as endothelial cell tissues, there is a particular need for new or improved drugs having
10 increased selectivity and specificity, and hence, reduced toxicities, for use in the treatment of several human disorders.

The present invention is directed to developing new
15 conjugates for use in targeting a compound or drug to vascular endothelial cells. The conjugates of the present invention were designed to have two components linked by a selectively-hydrolyzable bond, such as an acid-labile or enzyme-sensitive bond. The function of
20 the first component would be to specifically direct the conjugate to the target tissue, i.e. to the vascular endothelial cells. The second component is a selected agent, for example, a steroid, which would exert its effects only on the target cells following its release
25 from the targeting moiety at the cell surface or within intracellular compartments.

The targeting of the novel conjugates specifically to endothelial cells is an important feature of the
30 conjugates. This would enable various selected agents, including steroids, to be used whilst avoiding possible side-effects associated with their action on tissues other than the desired target tissue. This is a common occurrence, for example, the use of steroids is
35 associated with adverse effects on bone metabolism and the immune system.

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Compounds considered to be of use in targeting to vascular endothelial cells were naturally occurring and synthetic polyanionic compounds and polymers, and more particularly, polysulphated compounds and polymers.

5 Small polyanionic molecules of this kind are particularly exemplified by suramin and analogues and derivatives of suramin. Polymers of this kind include both *N*- and *O*-sulfated polyanionic polysaccharides such as heparin, heparan sulfate, chondroitin sulfate, keratan sulfate, 10 dermatan sulfate, sulfated chitin, sulfated chitosan, sulfated alginic acid, pentosan polysulfate and sulfated cyclodextrins or the like, or synthetic organic polymers such as polystyrene sulfonate, sulfated polyvinyl alcohol, polyvinyl sulfate or polyethylene sulfonate.

15

Suramin may be prepared as follows: 8-Amino-1,3,5-naphthalenetrisulfonic acid is condensed with *m*-nitro-*p*-toluoyl chloride in the presence of sodium acetate. The resulting nitro compound is reduced, and the amino 20 derivative is condensed with *m*-nitrobenzoyl chloride; the product is reduced to the corresponding amino compound which is then reacted with carbonyl chloride in the ratio of two moles of the former to one of the latter. The suramin acid thus obtained is neutralized with NaOH to 25 produce the sodium salt. Alternatively, it may be obtained from the CDC Drug Service, Centers for Disease Control, Atlanta GA 30333.

Suramin is known to be suitable for administration 30 to humans as it has been used in the treatment and prophylaxis of trypanosomiasis, and in the treatment of onchocerciasis, pemphigus, and in some cases, in the treatment of cancer and AIDS. The preferred mode of administering a suramin-containing conjugate is 35 contemplated to be by intravenous injection. From studies on the treatment of infectious diseases it is known that doses of 1 g per day administered on five days

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over a three week period are well tolerated by adults. Being a small molecule, it is considered that a conjugate containing suramin, or a derivative thereof, will likely be less immunogenic than a conjugate containing a polymer, and such conjugates may thus have some advantages as therapeutic agents in certain settings.

In one example, the component chosen to target the conjugates to the vascular epithelium was high molecular weight heparin. It was reasoned that a heparin derivative with intact O- or N-linked sulfate groups would bind to sulfated polyanion (heparin) receptors which are present on vascular endothelial cells (Florey et al., 1959; Hiebert & Jacques, 1976; Mahadoo et al., 1977; Bârzu et al., 1985; 1986; Pino, 1987; Van Rijn et al., 1987) and on cells of the reticuloendothelial system (Schaefer et al., 1980; Stau et al., 1973; Fabian et al., 1978).

The inventor also proposes to utilize a non-anticoagulating derivative of heparin, which would allow the normal clotting actions of the blood to occur. From structure-activity studies, distinct elements within the heparin molecule have been identified as being responsible for its various properties. The anti-coagulating activity has been shown to reside within the carboxyl groups of the first seven sugar moieties. Therefore the inventor proposed to modify such carboxyl groups whilst leaving the sulphate residues intact.

Many agents could be specifically delivered to vascular endothelial cells using a polyanionic targeting component as described above, including radioisotopes and drugs which inhibit or promote DNA synthesis or gene transcription. Particularly useful agents for conjugation include those which, for example, either stimulate or inhibit cellular proliferation.

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Pharmacological agents of this kind include ionophores, antimetabolites, autacoids, vinca alkaloids, epipodophyllotoxins, alkylating agents, phorbol esters, prostaglandins, antibiotic chemotherapeutic agents, and
5 in preferred embodiments, steroids, which are considered to be particularly useful as selected agents in the endothelial cell-targeted conjugates of the present invention.

10 Certain aspects of the present invention are particularly concerned with the creation of novel conjugates that can function as inhibitors of angiogenesis, for use in the treatment of diseases which depend on the inappropriate growth of blood vessels. To
15 create an angiogenesis inhibitor, the inventor first chose an anti-angiogenic steroid, such as cortisol (hydrocortisone). It was reasoned that the steroidal component of the conjugated inhibitor would act to specifically inhibit the proliferation of the vascular
20 endothelial cells, without otherwise damaging them. For example, cortisol would inhibit DNA synthesis, by altering gene transcription, and thereby suppress cell division.

25 Because steroids are known to exert their effects on cellular events by influencing gene transcription (Haynes, 1990) they have the potential to cause many unwanted disruptions to various processes in other cell types. By targeting the steroids specifically to
30 vascular endothelia, the present invention provides a means for limiting their effects specifically to such cells. Any undesirable effects on other tissues, such as bone and lymph, would be minimized by their limited exposure to the compound. In the case of the inhibitors,
35 it was contemplated that dividing vascular endothelial cells may also preferentially bind and endocytose more of

-40-

the conjugate, as had been observed for unconjugated heparin (Sakamoto & Tanaka, 1988).

5 The bond joining the two components of these novel
targeted conjugates was designed to be a selectively-
hydrolyzable bond, such as an acid-labile or enzyme-
sensitive bond. It was reasoned that the presence of
such a bond would allow the generally-stable conjugate to
be hydrolysed only under certain conditions, such as on
10 exposure to an acidic pH or to a specific enzyme. This
was considered to be a particularly important feature of
the conjugates, enabling them to be stable whilst in the
blood stream or other extracellular environments, and
allowing hydrolysis and the release of the selected agent
15 only inside the target cells.

 In designing an acid-labile bond, it is reasoned
that the endocytosis of the conjugate by the endothelial
cell would result in its delivery to acidified endosomes
and lysosomes. This is known to occur with heparin
20 itself (Fabian et al., 1978; Bärzu et al., 1985). The
presence of the acid-labile bond would then allow the
release of the selected agent from such acid
intracellular compartments. The selected agents, such as
25 steroids, would then be free to exert their effects only
when inside the target cells, and would be otherwise
maintained in an active state whilst circulating in the
body.

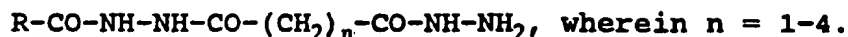
30 A variety of acid-labile bonds could be employed to
join the targeting component of a conjugate to the

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selected agent. For example, the inventor chose to utilize hydrazide linkers of the formulae:



5



10 The use of hydrazide linkers in conjugates is believed to provide certain advantages. For example, related heparin-steroid conjugates with linkers of different length, i.e. with differing numbers of CH_2 groups, could be prepared using the same synthetic protocol. This may influence the rate of conjugate hydrolysis and allow one
15 to engineer conjugates with different rates of release from the intracellular compartments, allowing a degree of control over the concentrations of active steroids in the cell.

20 Further acid-labile bonds that could be employed in accordance with the present invention incorporate ortho ester, acetal and ketal functionalities that undergo acid-catalyzed dissociation but are base-stable; and cis-aconitic, maleic, or citraconic acid functionalities
25 having a free carboxyl group juxtaposed to the acid-sensitive amide or ester linkage.

In other instances, it is proposed that conjugates with an enzyme-sensitive bond such as an ester, a
30 peptide, an amide, a phosphodiester or a glycoside will provide certain advantages. As this class of conjugates would be hydrolysed only by exposure to the specific enzyme, i.e. to an esterase, peptidase, an amidase, a phosphodiesterase or a glycosidase, this may allow
35 conjugates to be engineered that are hydrolysed only at the endothelial cell surface, or within the precise

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intracellular compartments where the specific enzymes are located.

One major finding that has emerged from the
5 inventor's studies clearly demonstrates that a particular
conjugate, a heparin-cortisol inhibitor, had all the
properties that were desired by the inventor. Namely, it
inhibited DNA synthesis and migration of vascular
endothelial cells *in vitro*, it retarded the
10 vascularization of sponges implanted into mice, and it
significantly inhibited the growth of solid Lewis lung
carcinomas in mice. In each assay, the activity of the
heparin-cortisol conjugate exceeded that of a mixture of
its components showing that, as reasoned beforehand,
15 chemical linkage of the components improved the
biological activity of the conjugate.

The heparin-cortisol conjugate was constructed by
first condensing the carboxyl groups of accessible
20 glucuronic acid and iduronic acid residues in heparin
with adipic dihydrazide. This resulted in the
introduction of hydrazide groups into heparin which, on
mixing with cortisol, condensed with the ketone group on
C3 of the steroid to form a conjugate in which the
25 heparin and the steroid were joined by an acyl hydrazone
bond. This bond was stable at pH 7.4 but, as required,
allowed the rapid dissociation of the conjugate at pH
4.8. The first destination of the conjugate following
internalization would be the acidified endosomes and
30 lysosomes, which, with a pH of ~4.8, would promote the
rapid release of the steroid.

Derivatization of heparin to obtain the hydrazide
destroyed the anticoagulant activity of heparin. This is
35 consistent with earlier reports concerning the synthesis
of heparinylglycine and heparinylglycine methyl ester
(Danishefsky & Siskovic, 1971). The inventor proposes

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that modification of either or both of the two iduronic acid residues in the pentasaccharide sequence near to the non-reducing terminus (Lindahl et al., 1983) distorted its anti-thrombin binding activity. Importantly, the synthetic procedure left the sulfate groups, which are important for tight endothelial cell binding, unaltered.

Heparin-cortisol inhibited DNA synthesis in cultured proliferating vascular endothelial cells at lower concentrations than did a mixture of its components. In addition, it delayed the repair of wounded HUVE monolayers, suggesting that, in addition to inhibiting cell proliferation, it inhibited endothelial cell migration. These two effects may explain why total abolition of vascularization was observed in sponges injected with heparin-cortisol. Vascularization is a complex process requiring both the migration of vascular endothelial cells into the sponge and then proliferation to enable the capillary sprout to elongate. Administration of unconjugated mixtures of heparin and cortisol, or heparin hydrazide and cortisol, did not affect vascularization of sponges.

The injection of heparin-cortisol into mice bearing solid Lewis lung carcinomas also resulted in statistically significant anti-tumor effects. Heparin-cortisol-treated mice showed a reduction in tumor mass of approximately 65% relative to that in untreated controls, and of approximately 40% relative to that in recipients of unconjugated heparin hydrazide and cortisol. A conjugate of heparin-adipic hydrazide and tetrahydro S was even more effective than was heparin-cortisol at inhibiting proliferation of vascular endothelial cells, indicating that the inhibitory effects of heparin-adipic hydrazide-cortisol are independent of the glucocorticoid and mineralocorticoid activities of the steroid.

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Tetrahydro S and related steroids offer certain advantages for use as conjugates *in vivo*. Being metabolically inert in all but angiostasis assays, they should not cause the toxic side effects (e.g. osteoporosis) associated with long term corticosteroid therapy. Long term treatment with heparin-adipic hydrazide conjugates is likely to be necessary in tumor therapy to suppress the outgrowth of dormant tumor cells. In addition, heparin-tetrahydro S inhibited DNA synthesis more potently and completely than did heparin-cortisol, suggesting that it should be a better anti-tumor agent *in vivo*. Unfortunately, tetrahydro S and related steroids lack a C3 ketone by which they can be conjugated efficiently to heparin-adipic hydrazide. Conjugation can be achieved through the C20 ketone, although this gives a lower yield (0.5 mol steroid per mol heparin-adipic hydrazide) than does cortisol conjugation (5 - 8 mol steroid per mol heparin-adipic hydrazide). The inventor is presently developing new, more efficient methods for forming heparin-adipic hydrazide-tetrahydro S.

The inventor proposes that promising results obtained to date may be improved upon further. It is possible that the inhibitory effects of systemically administered conjugates on tumor growth and sponge vascularization might be strengthened by a different dose regimen designed to increase and sustain blood levels. Since the effects of steroids on cell activation and/or proliferation are known to reverse rapidly when the steroid is removed, it is possible that a wave of endothelial cell proliferation occurred towards the end of each 24 hour interval between injections. If so, the problem should be solved simply by a continuous infusion of the conjugate. This can be investigated in animal models by employing an implanted mini-pump. If continuous infusion is found to be particularly advantageous in experimental animals, the inventor

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contemplates that biodegradable polymer-based drug-release capsules may be used clinically to allow the continuous supply of a conjugate.

5 As this invention provides a universally applicable means of targeting any drug or other component to a vascular endothelial cell, it is envisioned that novel conjugates of the present invention will find broad utility both *in vivo* and *in vitro*. Important medical
10 uses range from anti-tumor therapy and the treatment of diabetic retinopathy, to the control of inflammatory responses. It is envisioned that a wide variety of conjugates could be synthesized, using different targeting compounds or polymers and selected agents, for
15 use in the numerous clinical applications discussed above.

 Conjugates of the present invention are also contemplated to be of use in research, for example, in
20 investigating events such as the targeting, binding and uptake of polyanionic compounds or polymers, or in analyzing the specific effects of various components, including steroids, on endothelial cells. This includes analyzing the proliferation and migration of endothelial
25 cells in experimental animals treated with certain conjugates, and also, investigating the *in vivo* expression, and particularly, the cell surface expression, of various molecules induced by such treatment.

30 The novel conjugates of the present invention may be administered alone or in combination with pharmaceutically acceptable carriers, in either single or multiple doses. Suitable pharmaceutical carriers include
35 inert solid diluents or fillers, sterile aqueous solution and various organic solvents. The pharmaceutical compositions formed by combining a conjugate of the

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present invention and the pharmaceutically acceptable carriers are then easily administered in a variety of dosage forms such as injectable solutions, slow-release biodegradable capsules, and the like.

5

For parenteral administration, solutions of the conjugates in sesame or peanut oil, aqueous propylene glycol, or in sterile aqueous solution may be employed. Such aqueous solutions should be suitably buffered if
10 necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection,
15 sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure.

The following examples are included to demonstrate
20 preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus
25 can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result
30 without departing from the spirit and scope of the invention.

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EXAMPLE I
DERIVATIZATION OF HEPARIN

1. Materials.

5

Heparin (sodium salt; H3125; Grade 1 from porcine intestinal mucosa, 181 USP units/mg), adipic dihydrazide, cortisol (hydrocortisone; 11 β , 17 α , 21-trihydroxypregna-4-ene-3, 20-dione; H4001), tetrahydro S and Russell's
10 viper venom (RVCL-1, freeze-dried powder reconstituted to 3ml with saline) were from Sigma Chemical Co., St. Louis, MO. Carbazole, sodium tetraborate, EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) and other chemicals were from Aldrich Chemical Co., Milwaukee, WI. All
15 reagents were Analar or equivalent, or the best grade available.

Goat anti-human Von Willebrand factor (vWF) antibody was purchased from DAKO Ltd., High Wycombe, England.
20 ³H-thymidine and ¹³³Xe were from Amersham International, Amersham, U.K. Polyether-polyurethane sponge (VP45) was a gift from Mr. M. Robinson, Vitafoam Ltd., Ashton-under-Lyne, U.K. Disks of diameter 0.8cm were cut from sheets of the sponge using a cork-borer. The tissue culture
25 medium M199, with Earle's salts, was obtained from Gibco-Biocult Ltd., Paisley, Scotland; fetal calf serum was obtained from ICN Biomedicals, Inc., Costa Mesa, CA; tissue culture medium DMEM was obtained from Gibco BRL, Gaithersburg, MD; insulin-transferrin-sodium selenite
30 media supplement and endothelial cell growth supplement (0.12 mg/ml) were obtained from Sigma Chemical Co. Ninety-six well flat-bottomed microtiter plates and 24-well tissue culture plates were obtained from Falcon (Becton Dickinson & Co., Lincoln Park, NJ). MECA 20, a
35 pan anti-mouse vascular endothelial cell antibody (Duijvestijn et al., 1987), was obtained from Dr. Adrian

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Duijvestijn, Rijksuniversiteit Limburg, Maastricht, The Netherlands.

2. Measurement of Heparin Concentration.

5

The concentration of heparin solutions was determined by using a modification of the carbazole assay described by Bitter & Muir (1962). Briefly, the heparin solutions to be analyzed were diluted until their approximate concentration was in the range of 25-250µg/ml. 200µl of the heparin solutions were added dropwise to glass tubes containing 1ml of an ice-cold solution of sodium tetraborate (5.03mg/ml) in concentrated sulfuric acid. The solutions were mixed and heated at 95°C for 10 minutes. 40µl of carbazole (1.25mg/ml) in absolute ethanol was added to each tube and the solutions were mixed and heated at 95°C for 15 minutes. The tubes were allowed to cool and the optical density was measured at 530 nm. The heparin concentrations were calculated by comparing the optical densities of duplicate samples of the test solutions with those of standards prepared from heparin solutions having concentrations ranging from 0 to 250µg/ml.

3. Condensation of Heparin and Hydrazine.

Hydrazine (1.567 ml) was made up to 50 ml with 1M HCl, adjusted to pH 4.75 with 1M NaOH, and diluted to 100 ml. 20 ml of this solution was added to 200 mg of heparin, which dissolved on shaking. EDC in water (4 ml) was added to the hydrazine-heparin solution (20 ml) to give a EDC concentration of 10.7 mg/ml. The solution was adjusted at intervals to pH 4.75, dialyzed twice in 1mM HCl and once in 0.05M borate solution with NaCl (1.7% w/v), and then three times in distilled water and was freeze dried.

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4. Condensation of Heparin and Succinic Dihydrazide.

A solution of heparin (100 mg/ml) in distilled water was filtered through a column of sepharose S200 (5 x 60 cm) to remove low molecular weight heparin present in this material. The fractions containing the highest molecular weight components were pooled until 70% of the heparin applied to the column had been recovered, and the lower molecular weight fractions were discarded. Heparin was then condensed with succinic dihydrazide in a similar manner to that described for the synthesis of heparinylglycine (Danishefsky & Siskovic, 1971). The concentrate was adjusted to 10 mg/ml and adipic dihydrazide was added to a final concentration of 0.5M. The pH of the solution was adjusted to 4.75 using 1M HCl. A freshly prepared solution of EDC in water was added to give a final EDC concentration of 10.7 mg/ml.

The reaction was allowed to proceed at room temperature while maintaining the pH at 4.75 (± 0.02) by the periodic addition of 1M HCl. When the pH had stabilized (2-3 hours), the mixture was kept at 4°C for 48 hours and was then dialyzed (using tubing having a M_r cut-off of 3500) into 1 mM HCl in water at 4°C with four changes of dialysis buffer over 4 days. The retentate was then freeze dried.

5. Condensation of Heparin and Adipic Dihydrazide.

Low molecular weight heparin was removed from a solution of heparin (10 mg/ml) in distilled water by passing through an Amicon ultrafiltration cell fitted with a YM30 membrane until 30% of the heparin appeared in the filtrate. Heparin was then condensed with adipic dihydrazide in a similar manner to that described for the synthesis of heparinylglycine (Danishefsky & Siskovic, 1971). The concentrate was adjusted to 10 mg/ml and

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adipic dihydrazide was added to a final concentration of 0.5M. The pH of the solution was adjusted to 4.75 using 1M HCl. A freshly prepared solution of EDC in water was added to give a final EDC concentration of 10.7 mg/ml.

5

The reaction was allowed to proceed at room temperature whilst maintaining the pH at 4.75 (± 0.02) by the periodic addition of 1M HCl. When the pH had stabilized (2-3 hours), the mixture was kept at 4°C for 48 hours and was then dialyzed (using tubing have a M_r cut-off of 3500) into 1mM HCl in water at 4°C with four changes of dialysis buffer over 4 days. The dialyzed solution was again extensively dialyzed into deionized water at room temperature. The resulting aqueous solution was freeze-dried and was stored in desiccator at 4°C.

20

EXAMPLE II

CONJUGATION OF HEPARIN DERIVATIVES

1. Conjugation of Daunomycin and Carminomycin to the Adipic Hydrazide Derivative of Heparin.

25

Daunomycin and carminomycin contain amino sugars which interact electrostatically with heparin and cause a precipitate to form. To avoid this, the amino group was first converted to the cis-aconityl derivative which is removed at acidic pH (i.e., at the same time as the conjugated drug dissociates from the heparin carrier).

30

Daunomycin or carminomycin (20 mg) were dissolved in 4 ml of 0.05M sodium borate buffer, pH 9, and stored on ice. To this solution were dropwise added 200 μ l of a freshly-prepared solution of cis-aconitic anhydride (30 mg/ml) in dry dioxan. The reaction was allowed to proceed for 10 minutes on ice and then for a further 10

35

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minutes at room temperature. Thin layer chromatography on silican plates run in 3:1 ethanol:H₂O revealed that >90% of the daunomycin and carminomycin had been converted to their cis-aconityl derivatives having R_f values of 0.75 as compared with 0.05 for the unreacted drugs. The acid liability of the introduced cis-aconityl protecting group was confirmed by mixing the cis-aconityl carminomycin with an equal volume of 1M sodium acetate buffer, pH 4.3. Samples were run on silica TLC plates in MeOH. The half life of dissociation of the derivatives back to native drugs was approximately 60 minutes under these conditions.

Next, the cis-aconityl daunomycin and cis-aconityl carminomycin were conjugated to the adipic hydrazide derivative of heparin via the $\cdot\text{CO}\cdot\text{CH}_3$ group present in the drugs. Heparin adipic hydrazide (46.1 mg) was dissolved in 3 ml of 0.5M sodium acetate buffer, pH 6.0. To this solution was added 0.68 ml of a 9 mM solution of cis-aconityl drug in dry dioxan. The solutions were mixed and allowed to react for 24 hours at room temperature. The solutions were then applied to columns of Sephadex G25 equilibrated in H₂O whose pH had been raised to 9 with dilute NaOH. The void volume peak was collected and freeze dried. Spectrophotometric (u.v) quantitation of the coupled drugs together with measurements of heparin concentration by carbazole assay gave the drug:heparin ratios as 1.6 mol:mol for daunomycin and 0.6:1 for carminomycin.

30

To confirm that the drugs could be released from the heparin adipic hydrazide carrier at acidic pH, a study was performed in which samples of heparin-daunomycin conjugate were incubated at pH 4.3 or pH 3.3 for various periods of time. The samples were then analyzed using a TLC system in which silica plates were run in 3:1 H₂O:MeOH. In this system, the conjugate moves with an R_f

35

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of 0.6 whereas free daunomycin remains on the origin. Incubation of the conjugate at pH 3.3 at room temperature for 30 minutes caused complete dissociation of the conjugate to give free drug. By contrast, at pH 4.3, dissociation was slow: only about 10-20% of the conjugate dissociated within 4 hours. At pH 7, dissociation was undetectable. The daunomycin and carminomycin conjugates (Table III) have the structures shown in Fig. 1.

2. Conjugation of Mithramycin to the Adipic Hydrazide Derivative of Heparin.

Heparin adipic hydrazide (70 mg) was dissolved in 6 ml of 0.1M sodium phosphate buffer, pH 5.8. Mithramycin (10 mg) was dissolved in 1 ml dimethylformamide and this solution was mixed with the heparin adipic hydrazide solution and left for 2½ days at room temperature. The reaction mixture was then applied to a column of Sephadex G25 equilibrated in H₂O whose pH had been raised to approximately 10 by the addition of dilute NaOH. The conjugate, which eluted in the void volume, was collected and freeze dried. Quantitation of the drug by u.v spectrophotometric analysis and of the heparin by carbazole assay gave the composition of the conjugate as 0.54 mol mithromycin:mol heparin. The conjugate (Table III) has the structure shown in Fig. 2.

3. Conjugation of Aminopterin to the Adipic Hydrazide Derivative of Heparin

30

Direct Conjugation: Aminopterin (102 mg) was dissolved in 10 ml dimethyl formamide with gentle heating. To this solution was added 26.7 mg of N-hydroxysuccinimide dissolved in 0.315 ml dimethyl formamide, followed by 47.8 mg of dicyclohexylcarbodiimide dissolved in 0.523 ml of dimethylformamide. The mixture was allowed to react for 20 hours at room temperature. The urea that formed

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during the reaction was removed by centrifugation. Analysis by TLC on silica plates run in 1:1 methanol:H₂O showed that the product(s) ran with an R_f of 0.85 as compared with 0.96 for aminopterin and 0.93 for
5 *N*-hydroxysuccinimide.

To 27.3 ml of a 18.3 mg/ml solution of heparin adipic hydrazide in acetate buffer, pH 6.0, were added 6 ml of a 16 mM solution of the *N*-hydroxysuccinimide
10 derivative of aminopterin in dimethylformamide. The reaction was allowed to proceed for 24 hours at room temperature. The mixture was then applied to a column of Sephadex G25 equilibrated in H₂O. The conjugate, which eluted in the void volume, was collected and freeze
15 dried. Quantitation of the aminopterin content by u.v spectrophotometric measurements and of the heparin content by carbazole assay showed that the conjugate contained 1.66 mol aminopterin:mol heparin.

20 The conjugate (Table III) predominantly has the structure shown in Fig. 3 in which the aminopterin is linked through its γ COOH group to the heparin derivative.

25 Conjugation Via Leu-Ala-Leu-Ala Spacer: The *N*-hydroxysuccinimide ester of aminopterin was prepared as above. To 10.2 ml of a 23 mM solution of the aminopterin derivative (0.236 mmol) in dimethylformamide were added 104 mg (0.236 mmol) of solid L-Leu-L-Ala-L-Leu-L-Ala
30 (LALA). The mixture was stirred overnight at 50°C during which time the solid LALA dissolved. To the reaction mixture was added 48.7 mg (0.236 mmol) of dicyclohexylcarbodiimide in 0.58 ml of dimethylformamide. Copious quantities of urea formed within 2 hours at room
35 temperature and were removed by filtration. The filtrate containing the *N*-hydroxy succinimide ester of the LALA derivative of aminopterin was retained.

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To 500 mg of heparin adipic hydrazide at 19.3 mg/ml in 0.1M sodium acetate buffer, pH 6.0, were added 7.4 ml of a 13.5 mM solution of the N-hydroxysuccinimide ester of LALA-aminopterin in dimethylformamide. The reaction
5 was left for 24 hours at room temperature in the dark. The reaction mixture was applied to a column of sephadex G25 equilibrated in H₂O. The conjugate, which eluted in the void volume, was collected. Quantitation of
10 aminopterin content by u.v spectrophotometric measurements and of heparin content by carbazole assay showed that the conjugate contained 4.9 mol aminopterin:mol heparin.

The conjugate probably contained different species
15 depending on which carboxyl group was esterified with N-hydroxysuccinimide at each stage of the reaction. However, the predominant reaction is with the most reactive and accessible carboxyl group. Thus, the conjugate (Table III) should predominantly have the
20 structure shown in Fig. 4.

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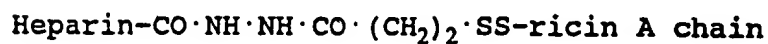
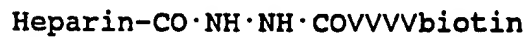
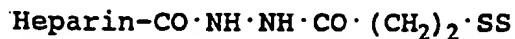
TABLE III
CONJUGATES OF HEPARIN AND CHEMOTHERAPEUTIC DRUGS

DRUG	LINKAGE	HEPARIN DERIVATIVE	MECHANISM OF HYDROLYSIS
*Dauno-mycin	Acyl hydrazone	Adipic hydrazide	acid-catalyzed
*Carmino-mycin	Acyl hydrazone	Adipic hydrazide	acid-catalyzed
Mithra-mycin	Acyl hydrazone	Adipic hydrazide	Acid-catalyzed
Aminop-terin	Amide	Adipic hydrazide	Amidase
Aminop-terin	Leu-Ala-Leu-Ala	Adipic hydrazide	Peptidase

* as cis-aconityl derivatives

4. Further Heparin Conjugates.

Heparin derivatives have also been conjugated to other selected agents, including 3-(2-pyridyldithio) propionic acid (PDP), biotin and ricin A chain, as represented below:



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The PDP derivative was prepared by treating 5 ml of a solution of heparin adipic hydrazide (5 mg/ml) in 0.05M sodium borate buffer, pH 9, with 0.2 ml of a solution of N-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (SPDP, Pharmacia Ltd.) (5.2 mg/ml) dissolved in dry dimethylformamide. The reaction was allowed to proceed for one hour at room temperature and the heparin derivative was then dialyzed into water. Quantitation of the introduced dithiopyridyl groups by the method of Carlsson et al. (Biochem. J. 173, 723, 1978) and of the heparin by carbazole assay gave the molar ratio of dithiopyridyl groups:heparin as 1:2:1.

The biotin derivative of heparin adipic hydrazide was prepared by treating 1 ml of a solution of heparin adipic hydrazide (5 mg/ml) in 0.05M sodium borate buffer, pH 9, with 0.2 ml of a solution of N-hydroxysuccinimidyl biotin amidocaproate (3.8 mg/ml) dissolved in dry dimethylformamide. The reaction was allowed to proceed for one hour at room temperature and the heparin-biotin conjugate was then dialyzed into water.

Ricin A-chain was conjugated to heparin adipic hydrazide by mixing 5 ml of a 1 mg/ml solution of PDP-heparin (prepared as above) with 10 mg of freshly-reduced ricin A-chain in phosphate-buffered saline, pH 7.4. The thiol-disulfide exchange reaction leading to conjugation was allowed to proceed for 24 hours. The conjugate was fractionated on a column (2.2 x 80 cm) of Sephadex G75 and the leading edge of the major protein band which eluted was collected. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS/PAGE) showed that the conjugate mainly (70%) consisted of a component approximately 50 kDa corresponding to one molecule of heparin coupled to one molecule of ricin A-chain.

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5. **Conjugation of Different Steroids to Heparin Adipic
Hydrazide.**

A solution of heparin adipic hydrazide (1.0 mg/ml)
5 in 0.025M sodium acetate buffer pH 3.0 was prepared. A
solution of the steroid in ethanol was added to give a
final molar excess of steroid over hydrazide groups of
10-fold, and a final ethanol concentration of 30% (v/v).
The steroid and the heparin adipic hydrazide were allowed
10 to react at different temperatures and for different
periods of time depending on the reactivity of the ketone
groups of the steroid. The reaction conditions were:

- a) 48 hours at room temperature;
- 15 b) 72 hours at room temperature with stirring;
- c) 7 days at room temperature;
- d) 10 days at 56°C.

The reaction conditions employed for the synthesis
20 of conjugates with different steroids are listed in Table
IV.

After the reaction, the pH of the mixture was
adjusted to pH 10 with 10M NaOH. The heparin-steroid
25 solution was then dialyzed extensively against water
whose pH had been adjusted to 10 with a few drops of 1M
NaOH. The heparin-steroid conjugate was finally freeze
dried and stored in a desiccator at 4°C.

30 6. **Conjugation of Tetrahydro S to Heparin Adipic
Hydrazide.**

A heparin-tetrahydro S conjugate was prepared
according to the methodology described above in section
35 5. 7 volumes of heparin adipic hydrazide (1.0 mg/ml) in
0.025M sodium acetate buffer, pH 4.0, were mixed with 3
volumes of tetrahydro S at 10.4 mg/ml in ethanol. The

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mixture was heated at 56°C for 14 days and then was adjusted to pH 10, dialyzed and freeze-dried as before. The molar ratio of tetrahydro S to heparin-adipic hydrazide in the resultant conjugate was calculated by determining the content of heparin-adipic hydrazide in the conjugate by the carbazole assay as above and the content of tetrahydro S by releasing the steroid under acidic conditions and measuring the released steroid using the blue tetrazolium assay described by Chen et al. (1953). In brief, 1 ml of a 10 mg/ml solution of HAH-tetrahydro S in H₂O was dialyzed twice for 2-3 days at room temperature into 10 ml of 0.01 M acetic acid solution in H₂O. The dialyzates were combined and freeze dried. The dry residue was dissolved in 5 ml ethanol. To 2 ml of the ethanol solution were added 1 ml of a solution of 0.14 ml 5 M NaOH in 25 ml ethanol and 2 ml of a 2.5 mg/ml solution of blue tetrazolium in ethanol. After 1 hour at room temperature, the optical density of the solution was measured at 510 nm and the steroid concentration was calculated by reference to the absorption of a set of standard steroid solutions prepared and assayed on the same occasion.

The product consisted of 0.5 mol tetrahydro S per mol heparin-adipic hydrazide. Increasing the temperature or the duration of the reaction, or increasing the tetrahydro S concentration in the reaction mixture, did not result in an improved level of loading. The product is similar in structure to that shown in Figure 5 except that the heparin-adipic hydrazide forms an acyl hydrazone bond with C20 of the steroid rather than with C3.

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7. Conjugation of Different Steroids to the Hydrazine and Succinic Dihydrazide Derivatives of Heparin (Method (e)).

5 The condensation products of heparin and hydrazine
on succinic dihydrazide were dissolved in 0.5M sodium
acetate buffer pH 4.0 at 4.9 mg/ml. Aqueous solutions or
suspensions of steroids were added in 10-20 fold molar
excess over hydrazide groups and the mixture stirred for
10 24 hours at room temperature. The mixture was then
adjusted to pH 9 with 10M NaOH. The conjugate was
separated from unconjugated steroid by gel filtration on
a column of sepharose G25 equilibrated in water whose pH
had been adjusted to 10 with 1M NaOH. The fractions
15 containing the conjugate were pooled and freeze dried.

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TABLE IV
EXAMPLES OF HEPARIN-STEROID CONJUGATES

	STEROID	HEPARIN DERIVATIZATION	LINKAGE METHOD
5	Cortisol	Adipic dihydrazide	(a)
	17 α -hydroxyprogesterone	Adipic dihydrazide	(a)
	Cortisone	Adipic dihydrazide	(a)
	Corticosterone	Adipic dihydrazide	(a)
10	Deoxycorticosterone	Adipic dihydrazide	(a)
	Progesterone	Adipic dihydrazide	(a)
	Testosterone	Adipic dihydrazide	(a)
	Pregnenolone	Adipic dihydrazide	(a)
	Cortexolone	Adipic dihydrazide	(b)
15	Estrone	Adipic dihydrazide	(b)
	Tetrahydrocortisone	Adipic dihydrazide	(c)
	Tetrahydrocorticosterone	Adipic dihydrazide	(c)
	Tetrahydrocortisol	Adipic dihydrazide	(d)
20	Tetrahydro S	Adipic dihydrazide	(d)
	Dexamethasone	Adipic dihydrazide	(d)
	Cortisol	Hydrazine	(e)
	Cortisol 21-phosphate	Hydrazine	(e)
25	Cortisol 21-hemisuccinate	Hydrazine	(e)
	Dexamethasone	Hydrazine	(e)
	Dexamethasone 21-phosphate	Hydrazine	(e)
30	Dexamethasone 21-phosphate	Succinic dihydrazide	(e)

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EXAMPLE IIISTRUCTURE AND PROPERTIES OF HEPARIN-CORTISOL CONJUGATES

5 This example relates largely to the structure and chemical properties of the conjugate formed between cortisol and heparin adipic hydrazide.

1. Preparation.

10 Heparin, from which the low molecular weight species had been removed, was condensed with adipic dihydrazide in a similar manner to that employed in the synthesis of heparinylglycine (Danishefsky & Siskovic, 1971), as described above in Example I, Section 5. The heparin
15 adipic hydrazide was then dialyzed into 0.1M sodium acetate buffer, pH 4.0 and this solution adjusted to 1mg/ml by addition of 0.1M sodium acetate buffer, pH 4.0.

The conjugation reaction was performed as outlined
20 in Example II, Section 5. A solution of cortisol (11mg/ml) in ethanol was added to the heparin-hydrazide solution to give a final cortisol concentration of 3.2mg/ml and a final ethanol concentration of 30% (v/v). The mixture was allowed to react for 16 hours at room
25 temperature and was then adjusted to pH 10 with 1M NaOH. The resultant heparin-cortisol was dialyzed extensively into water whose pH had been adjusted to 10 with a few drops of 1M NaOH. The dialyzed product was finally freeze-dried and stored in a desiccator at 4°C.

30

2. Structure.

The molar ratio of cortisol to heparin in this product was determined by measuring the bound cortisol
35 and heparin concentrations in a standard solution. The heparin concentration was determined using the carbazole method described above and the molar concentration was

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calculated assuming an average molecular weight of 15,000 Da (Bârzu et al., 1986). The cortisol concentration was measured spectrophotometrically at 274 nm, using an extinction coefficient of $21,900 \text{ M}^{-1}$ for the acyl
5 hydrazone derivative of cortisol. This extinction coefficient was determined by dissociating samples of heparin-cortisol at acidic pH and measuring the released cortisol concentration from the optical density at 242 nm (using the extinction coefficient of $16,100 \text{ M}^{-1}$ (Budavari,
10 1989) after correcting for 242 nm absorbance due to the heparin-hydrazide. The molar ratio of cortisol:heparin in the product was usually found to be between 8 and 9.

The structure of this heparin-cortisol product is
15 schematically represented in Fig. 5. There are two ketones in cortisol which could have potentially reacted with the hydrazide, one at C3 on the A-ring and the other at C20 on the side chain. However, significant reaction only appears to have occurred with the C3 ketone, under
20 the conditions used in the present study, to give the product depicted in Fig. 5. This is evident from the fact that the chromophoric A-ring of cortisol, which has a UV spectrum having a λ_{max} at 242 nm, changes on reaction with hydrazides to a product having a λ_{max} at 274 nm: a
25 change in the absorption properties of the A-ring would not be expected if the linkage were with the C20 ketone.

3. Acid Lability.

30

0.75ml of a solution of a heparin-cortisol conjugate (200 $\mu\text{g/ml}$) in distilled water was added to a 1.5ml spectrophotometer cell in a thermostatically-controlled holder maintained at 20°C or 37°C. For analysis at pH
35 4.8, 0.75ml of 0.1M sodium acetate, pH 4.8, was added and the contents of the cell were rapidly mixed. The UV spectrum from 400 nm to 200 nm was scanned immediately

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and every 5 to 15 minutes thereafter until the decomposition of the heparin-cortisol was complete. The half-life of the decomposition was taken as the time for the OD at 274 nm to decay to half its initial value.

5

The dissociation of this heparin-cortisol conjugate is represented in Fig. 6, and the half-lives of dissociation shown in Table V. Data obtained for two heparin-cortisol phosphate conjugates are also presented (Table V, Fig. 7). At pH 7.4, the conjugate formed between cortisol and heparin adipic hydrazide was relatively stable: only 8% breakdown had occurred after 24 hours at 20°. However, this conjugate broke down to give free cortisol and heparin-hydrazide at pH 4.8, with a half-life of 35 minutes at 18°C to 20°C, and 15 minutes at 37°C (Table V, Fig. 6). The conjugate was as stable when incubated at 37°C in complete tissue culture medium which had been conditioned by MPCE cells as it was in PBS.

20

TABLE V
HALF-LIVES OF DISSOCIATION OF
HEPARIN-CORTISOL CONJUGATES AT pH 4.8

25	STEROID	HEPARIN DERIVATIVE	TEMPERATURE	T _{1/2} (minutes)
	Cortisol	Adipic dihydrazide	18°C	35
	Cortisol	Adipic dihydrazide	37°C	15
	Cortisol phosphate	Adipic dihydrazide	18°C	35
30	Cortisol phosphate	Hydrazine	18°C	90

These results demonstrate that the acyl hydrazone bond joining the two components of the conjugate was

35

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markedly acid-labile, as expected. Uptake of heparin-cortisol by endothelial cells and delivery of the conjugate to acidified endosomes and lysosomes would thus be expected to lead to the rapid release of the steroid since these intracellular compartments have a pH of approximately 4.8.

4. Anticoagulant Activity.

The anticoagulant activities of heparin, heparin-hydrazide, and the resultant heparin-cortisol conjugate were measured by their ability to inhibit coagulation of human plasma by Russell's viper venom. 20 μ l solutions of heparin, heparin-hydrazide or heparin-cortisol at a range of concentrations from 1-1000 μ g/ml in phosphate-buffered saline (PBS) were added to glass tubes which had been pre-warmed to 37°C. Fresh citrated human plasma (150 μ l) at 37°C was added to each tube. The contents were immediately mixed and the tubes were incubated at 37°C for 1 minute. One hundred microliters of Russell's viper venom (Sigma# RVCL-1 reconstituted to 3ml) were added and the contents were mixed. After 15 seconds, 100 μ l of a prewarmed solution of CaCl₂ (0.2M) in PBS were added. The contents of the tubes were immediately mixed and the tubes were incubated at 37°C. The time for the plasma to clot was recorded. The clotting times for plasma treated with heparin-hydrazide or heparin-cortisol were compared to those treated with unmodified heparin and the differences in anticoagulant activities were calculated.

30

Heparin hydrazide and the heparin-cortisol conjugate formed therefrom were found to be virtually devoid of anticoagulant activity, having approximately 1% of the anticoagulant activity of native heparin, i.e. about 2 USP units/mg. This destruction of anticoagulant activity on derivatization is in agreement with results reported by Danishefsky and Siskovic (1971) for heparinylglycine

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and heparinylglycine methyl ester. Presumably, modification of either or both of the two iduronic acid residues in the pentasaccharide sequence near to the non-reducing terminus (Lindahl et al., 1983) distorts its anti-thrombin binding activity. The absence of anti-coagulant activity of the product is advantageous because hemorrhage is unlikely to be a problem when heparin-cortisol is administered to animals. The heparin-cortisol should, however, retain its ability to bind to endothelial cells because the sulfate groups which are important for tight binding are unaltered.

EXAMPLE IV

IN VITRO INHIBITION OF DNA SYNTHESIS AND WOUND REPAIR BY HEPARIN-STEROID CONJUGATES

The present example, and those following (Examples V and VI), relate to the action of the conjugates formed between heparin adipic hydrazide and either cortisol or tetrahydro S.

1. Methods

a) DNA Synthesis by Human Vascular Endothelial Cells In Vitro.

Human umbilical vein endothelial cells (HUVE) were isolated from fresh human umbilical cords as described by Jaffe et al., (1973). Cultures were maintained at 37°C in an atmosphere of 5% CO₂ in humidified air in Medium 199 supplemented with Earle's salts, 20% (v/v) fetal calf serum (FCS), endothelial growth supplement (0.12mg/ml), 0.09mg/ml heparin, 2.4mM L-glutamine, 200 units/ml penicillin and 100µg/ml streptomycin. Cells were cultured in tissue culture flasks precoated with 1% (w/v) gelatin and were used at the third or fourth passage.

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Cultures were judged to be > 95% endothelial cells by morphological criteria and by positive staining of monolayers with monoclonal anti-vWF antibody, F8/86 (Naieum et al., 1982).

5

Studies designed to determine inhibition of DNA synthesis were performed as follows: HUVE cells were removed from confluent tissue culture flasks with trypsin (0.025% w/v) and EDTA (ethylenediaminetetraacetic acid, 10 0.02% w/v). A cell suspension was prepared at 10^4 cells/ml in culture medium containing all supplements with the exception of heparin. The suspension was distributed in 100 μ l aliquots into the wells of 96-well flat bottomed microtiter plates which had precoated with 15 1% (w/v) gelatin in water. The cells were incubated for 24 hours at 37°C during which time they adhered to and spread onto the surface of the wells. 100 μ l solutions of heparin-cortisol or various control substances dissolved in heparin-free tissue culture medium were then added and 20 the cells were incubated for 24 hours at 37°C. Each culture was then pulsed with 1 μ Ci 3 H-thymidine for 24 hours. The cells were washed with PBS, detached with trypsin-EDTA, and harvested onto glass fiber disks using an automated cell harvester. Acid insoluble 25 radioactivity in treated cultures was expressed as a percentage of that in cultures treated with medium alone to obtain the % inhibition of DNA synthesis. All cultures were set up in triplicate.

30 b) DNA Synthesis by Murine Vascular Endothelial Cells
In Vitro.

MPCE cells were obtained from Professor Adam S.G. Curtis, University of Glasgow, U.K. The cells are a 35 pulmonary capillary endothelial cell line isolated from B10.D2 mice. They are vWF positive, LDL receptor positive, synthesize collagen type III, are Gryphonina

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lectin positive and have endothelial morphology. The cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) fetal calf serum, 1% (v/v) insulin-transferrin-sodium selenite (ITS) media supplement, 2 mM L-glutamine, and 2 units/ml Penicillin G and 2 μ g/ml streptomycin. Cultures were maintained at 37°C in an atmosphere of 10% CO₂.

Studies designed to determine inhibition of DNA synthesis in MPCE cells were performed, in quadruplicate, essentially as described above for HUVE cells. MPCE cells were removed from tissue culture flasks with trypsin (0.625% w/v) and EDTA (0.2% w/v). A cell suspension was prepared at 5×10^4 cells/ml in culture medium containing 10% serum and supplements as above. The cell suspension was dispensed in 100 μ l aliquots into the wells of 96-well flat bottomed microliter plates. The cells were incubated for 24 hours at 37°C during which time they adhered to and spread onto the surface of the wells. 100 μ l solutions of heparin-adipic hydrazide-cortisol or various control substances, such as cortisol, heparin-adipic hydrazide, or a mixture of heparin-adipic hydrazide and cortisol, dissolved in tissue culture medium were then added to designated wells and the cells were incubated for 24 hours at 37°C. All cultures were set up in quadruplicate. Each culture was then pulsed with 1 μ Ci ³H-Tdr for 24 hours. The cells were washed with PBS, detached with trypsin-EDTA, and harvested onto glass fiber disks using an automated cell harvester. Radioactivity in treated cultures was expressed as a percentage of that in cultures treated with medium alone to obtain the percent inhibition of DNA synthesis.

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c) DNA Synthesis by 3LL Cells In Vitro.

Studies designed to measure inhibition of DNA synthesis were performed in a manner similar to that described above for HUVE cells. 100 μ l of a suspension of 3LL cells at 4x10⁴ cells/ml in complete medium were distributed into 96-well microtiter plates, and incubated for 24 hours at 37°C. 100 μ l of solutions of heparin-cortisol or various control substances were then added and cultures were incubated for 24 hours at 37°C, 1 μ Ci/culture of ³H-thymidine was added and the quantity of label incorporated into TCA-insoluble material was determined.

d) Repair of Wounded Endothelial Cell Monolayers.

The culture medium above confluent HUVE cultures (in 24-well tissue culture plates) was exchanged for medium containing all supplements except heparin. The cells were cultured for 2 days and were then wounded with a plastic micropipet tip (Eppendorf). The wounds were approximately 0.5mm wide and 5-7mm long. To the wounded monolayer cultures was added heparin-cortisol at a final concentration of 10⁻⁵M with respect to heparin (1.4x10⁻⁴M with respect to cortisol). Other wounded cultures were treated with heparin-hydrazide (10⁻⁵M) alone, cortisol alone (1.4x10⁻⁴M), or with an unconjugated mixture of both. The cultures were incubated at 37°C for 5 days and were photographed.

30

Similar studies were also conducted with MPCE cells. MPCE cells were cultured in 6 well tissue culture plates, grown to confluence and wounded with a plastic pipet tip (Oxford Universal large tip) to create a wound approximately 3 cm long and 0.15 cm wide. The wounded cultures were either treated with heparin-adipic hydrazide-cortisol conjugate at a final concentration of

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2X10⁻⁴ (with respect to cortisol) or with an unconjugated mixture of heparin-adipic hydrazide (at a final concentration of 2.4X10⁻⁵M) plus cortisol (at a final concentration of 2X10⁻⁴M). The cultures were incubated at 37°C for 5 days and photographed.

2. Results and Discussion

a) Inhibition of HUVE Cell DNA Synthesis by Heparin-Cortisol.

At concentrations below 4x10⁻⁴M with respect to cortisol, heparin-cortisol inhibited DNA synthesis by HUVE cells to a significantly greater extent than did free cortisol or an unconjugated mixture of heparin hydrazide plus cortisol (Fig. 8a). At 1.6x10⁻⁴M with respect to cortisol (2x10⁻⁵M with respect to heparin-hydrazide), the conjugate inhibited DNA synthesis by 50%, whereas, at this same concentration, neither free cortisol nor the heparin-hydrazide plus cortisol mixture were inhibitory. However, at higher concentrations, the inhibitory effect of free cortisol surpassed that of the heparin-cortisol conjugate. At 6x10⁻⁴M with respect to cortisol, the inhibitory effect of heparin-cortisol reached a maximum of 70%, whereas at the same concentration, free cortisol inhibited DNA synthesis by 90%. This suggests that the uptake pathway for heparin-cortisol had become saturated at this concentration whereas that for cortisol had not. Heparin hydrazide was not inhibitory over the concentration range shown in Fig. 8a.

To confirm that the heparin-cortisol conjugate was binding to heparin receptors on HUVE cells, the study was repeated with the inclusion of competing levels of free native heparin (10⁻³) in the cultures. The presence of the competing ligand abolished the inhibitory effect of

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heparin-cortisol showing that, as expected, the effects of the conjugate were mediated through binding to heparin receptors.

5 b) Inhibition of MPCE Cell DNA Synthesis by Heparin-Cortisol.

At low concentrations, heparin-adipic hydrazide-cortisol inhibited DNA synthesis by MPCE cells to a significantly greater extent ($P < 0.001$) than did free cortisol, heparin-adipic hydrazide, or an unconjugated mixture of heparin-adipic hydrazide and cortisol (Fig. 8b). The conjugate inhibited DNA synthesis by 50% at a concentration of $10^{-5}M$ with respect to cortisol ($1.25 \times 10^{-6}M$ with respect to heparin-adipic hydrazide). At this same concentration free cortisol, free heparin-adipic hydrazide and the unconjugated mixture were all without inhibitory effect. A consistent finding was that heparin-adipic hydrazide-cortisol failed to inhibit DNA synthesis completely at high concentrations (i.e. $3 \times 10^{-4}M$ or greater with respect to cortisol) suggesting that the uptake mechanism for the conjugate becomes saturated at these concentrations. As shown in Figure 8b, the maximum inhibition of DNA synthesis induced by HAH-cortisol was 65%. Similar results to these in Figure 8b were obtained in 4 separate experiments.

c) Inhibition of MPCE Cell DNA Synthesis by Heparin-Tetrahydro S.

Tetrahydro S, a steroid which lacks glucocorticoid or mineralocorticoid activity, yielded a heparin-adipic hydrazide conjugate which was 3-fold more potent at inhibiting DNA synthesis by MPCE cells *in vitro* than was heparin-adipic hydrazide-cortisol. Treatment of the cells with heparin-adipic hydrazide-tetrahydro S at $3 \times 10^{-6}M$ with respect to steroid reduced their DNA synthesis by

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50% (Figure 8c) as compared to 10^{-5} M for heparin-adipic hydrazide-cortisol. The HAH-tetrahydro S conjugate was at least 20-fold more inhibitory than an unconjugated mixture of heparin-adipic hydrazide and tetrahydro S which had to be applied at 10^{-4} M with respect to steroid for 50% inhibition in DNA synthesis to result. The maximum inhibition of DNA synthesis obtained with high concentrations of heparin-adipic hydrazide-tetrahydro S exceeded that obtained with heparin-adipic hydrazide-cortisol. Treatment of the cells with HAH-tetrahydro S at 10^{-4} M with respect to steroid reduced the DNA synthesis of MPCE cells by more than 95% (Figure 8c) whereas heparin-adipic hydrazide-cortisol at 3×10^{-4} M with respect to steroid only reduced it by about 65%. Similar results to those depicted in Figure 8c were obtained in three separate experiments.

d) Inhibition of 3LL Cell DNA Synthesis by Heparin-Cortisol.

In contrast with HUVE cells, Lewis lung carcinoma (3LL) cells were relatively insensitive to heparin-cortisol. Treatment of 3LL cells with heparin-cortisol at 10^{-3} M with respect to cortisol reduced their DNA synthesis by only 36%. This same level of inhibition of HUVE cells was obtained with a 40-fold lower concentration of heparin-cortisol. Heparin-cortisol was also less inhibitory than was free cortisol or cortisol mixed with heparin hydrazide, which again contrasted with HUVE cells. When applied at 10^{-3} M with respect to cortisol, these agents inhibited DNA synthesis by 65 and 69% respectively. Thus, uptake of cortisol by 3LL cells is hindered rather than facilitated by its attachment to heparin, presumably because these cells lack receptors to bind the heparin moiety.

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e) Inhibition of Repair of Wounded Endothelial Cell Monolayers.

Heparin-cortisol retarded the repair of wounded HUVE cell monolayers. In wounded monolayers treated with free cortisol ($1.6 \times 10^{-4} \text{M}$) or with an unconjugated mixture of heparin-hydrazide plus cortisol ($1.6 \times 10^{-4} \text{M}$), migration of HUVE cells into the wounded area began within 24 hours and was complete by 72 hours. By contrast, in wounded monolayers treated with heparin-cortisol at $1.6 \times 10^{-4} \text{M}$ with respect to cortisol, little wound repair was evident by 5 days. This indicates that, in addition to inhibiting DNA synthesis, heparin-cortisol inhibits the migration of HUVE cells. Importantly, the cells in the unwounded areas of the culture remained morphologically healthy showing that heparin-cortisol inhibited migration and proliferation but was not toxic to quiescent cells.

Likewise, heparin-adipic hydrazide-cortisol retarded the healing of wounded MPCE murine vascular endothelial cell monolayers. In wounded monolayers treated with an unconjugated mixture of HAH ($2.4 \times 10^{-5} \text{M}$) and cortisol ($2 \times 10^{-4} \text{M}$), migration of MPCE cells into the wounded area began within 24 hours and was complete by 72 hours. In contrast, the wound was still evident on day 5 in monolayers treated with heparin-adipic hydrazide-cortisol at an equivalent concentration

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EXAMPLE VANTI-ANGIOGENIC EFFECTS OF HEPARIN-CORTISOL IN VIVO1. Methodsa) Vascularization of Implanted Sponges.

Measurements of the extent of vascularization of sponge implants in mice were made using the method of Andrade et al., (1987) with slight modifications. Sterile polyether-polyurethane sponge disks (0.8cm diameter x 0.4cm thickness) were prepared which contained a 1.2cm length of polypropylene tubing (1.4mm external diameter) protruding from the center of one circular face of the sponge and located 0.2cm deep into the sponge (i.e., midway through its thickness). The cannula was sewn into the sponge with silk sutures and its open end was sealed with a removable plastic plug. Male BALB/c mice aged 8-12 weeks were anesthetized and the sponges were aseptically implanted into a subcutaneous pouch which had been fashioned with curved artery forceps through a 1cm long dorsal midline incision. The cannula was exteriorized through a small incision in the subcutaneous pouch.

Vascularization of the sponge was assessed twice weekly after implantation by anesthetizing the mice and injecting 10 μ l of a sterile solution of ¹³³Xe in saline (5 x 10⁵ dpm/ml) into the sponge via the cannula. The cannula was immediately plugged to prevent escape of the gas. A collimated gamma scintillation detector was then situated 1cm above the sponge and the radioactivity was measured for 5 seconds each minute for 9 minutes. The radioactivity-versus-time data were fitted to an exponential decline curve using a least-squares regression algorithm and the half-life of clearance was computed. In freshly implanted avascular sponges the

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¹³³Xe was cleared slowly with a half-life of about 25 minutes whereas in a fully vascularized sponge the ¹³³Xe was cleared rapidly with a half-life of about 7 minutes. Normally, full vascularization of an implanted sponge
5 occurred in about 16 days.

Two types of studies were performed in which the effect of heparin-cortisol on vascularization of sponges was determined. In the first, heparin-cortisol (0.78mg)
10 in 25 μ l saline was injected into the sponge via the cannula every day for 10 days starting the third day after implantation. Other groups of mice received equivalent quantities of heparin-hydrazide (0.65mg), cortisol (0.13mg) or an unconjugated mixture of both. In
15 the second series of studies, mice received intraperitoneal injections of heparin-cortisol (2.35mg) or heparin-hydrazide (1.95mg) plus cortisol (0.39mg) in 0.25ml saline every day for 5 days starting on the third day after implantation, and then half these doses for the
20 next 7 days. Each treatment group contained 10 mice. Effects on vascularization were judged from changes in ¹³³Xe clearance rate. Differences in clearance rates were tested for statistical significance using Willcoxon's log-rank test (Swinscow, 1980) and the Mann-Whitney-
25 Wilcoxon (1976) Test for Two Independent Samples.

b) Enumeration of Blood Vessels and Endothelial Cells in Implanted Sponges.

30 A study was performed in which heparin-cortisol (1mg) was injected daily into implanted sponges via the cannula for 10 days starting 3 days after implantation. Other mice received equivalent doses of heparin hydrazide (0.83mg), cortisol (0.17mg), or of an unconjugated
35 mixture of heparin hydrazide (0.83mg) plus cortisol (0.17mg). A further group received an unconjugated mixture of native heparin (0.83mg) plus cortisol

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(0.17mg). Sponges were dissected out and transverse frozen sections were cut from half way through the sponge's thickness. Endothelial cells and blood vessels were identified by indirect immunoperoxidase staining with goat anti-human vWF antibody which crossreacts with mouse vWF and with a rat monoclonal antibody, MECA 20, which reacts with mouse vascular endothelial cells (Duijvestijn et al., 1987). The developing antibodies were horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin and rabbit anti-rat immunoglobulin respectively and were used according to the manufacturer's recommended procedures. Blood vessels were visible as vWF-positive structures with discernable lumens. About half of the vessels contained erythrocytes. Endothelial cells were visible as vWF-positive cells having the morphology of endothelial cells but lacking an adjacent lumen. The vessels and endothelial cells present in 15 microscope fields (in a diametric line across the section) were counted.

2. Results

Inhibition of Vascularization of Implanted Sponges.

Daily injections of heparin-cortisol (0.78mg) into implanted sponges in mice abolished vascularization of the sponge. This was evident from the fact that (i) the ¹³³Xe clearance rate of the heparin-cortisol-treated sponges remained the same as that of freshly implanted (avascular) sponges for as long as the injections were continued (Fig. 9a), and (ii) frozen sections of sponges from mice that had received 10 daily injections of heparin-cortisol were completely devoid of endothelial cells or blood vessels, as identified by anti-vWF staining (Table VI).

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The ^{133}Xe clearance rate of sponges injected with heparin-adipic hydrazide-cortisol was actually slower than that of freshly implanted (avascular) sponges. The same was true of sponges in mice receiving heparin-adipic hydrazide-cortisol intraperitoneally at the 7 day and 10 day post-implantation time points. Thus it appears that heparin-adipic hydrazide-cortisol changes processes besides vascularization which determine the ^{133}Xe clearance rate of the sponge. Possibly, heparin-adipic hydrazide-cortisol increases the viscosity of the fluid retained in the sponge and thereby retards escape of the gas.

Heparin-cortisol was the only substance which retarded the ^{133}Xe clearance rates. In control groups which received equivalent quantities of heparin hydrazide alone, cortisol alone, or a mixture of the two, ^{133}Xe clearance rates were the same as in mice which received saline alone (Table VI). However, the number of blood vessels and endothelial cells present in sections of sponges which had been injected with heparin hydrazide, either alone or mixed with cortisol, were reduced by about 45% and 65% respectively as compared with sponges injected with saline alone (Table VI). It is unclear why reductions in the number of blood vessels and endothelial cells in sponges injected with heparin hydrazide did not give rise to reductions in ^{133}Xe clearance rate. Conceivably, ^{133}Xe clearance rates are a fairly insensitive indicator of vascularization and major differences in vascularity are needed for perceptible differences in ^{133}Xe clearance rates.

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TABLE VI
ABOLITION OF VASCULARIZATION OF IMPLANTED SPONGES BY
DAILY INJECTIONS OF HEPARIN-CORTISOL INTO THE SPONGE

Material injected ^(a)	Number in 15 microscope fields ^(b)	
	Blood vessels ^(c) (with lumens)	Endothelial cells ^(c) (without lumens)
Saline	96 ± 22	96 ± 22
Heparin-cortisol conjugate	0	0
Heparin hydrazide	34 ± 18	31 ± 14
Cortisol	47 ± 12	74 ± 10
Heparin hydrazide plus cortisol	33 ± 13	35 ± 6
Native heparin plus cortisol	52 (41-63)	42 (35-48)

(a) Doses were 1.0mg heparin-cortisol, 0.83mg heparin hydrazide, 0.17mg cortisol, 0.83mg heparin hydrazide plus 0.17mg cortisol, or 0.83mg native heparin plus 0.17mg cortisol.

(b) Fifteen fields were counted which formed a straight line from the edges to the center of each sponge. The numbers given are the average number of vessels and endothelial cells in all 15 fields from sponges taken from 4 to 7 mice ± one standard deviation. In the case of native heparin plus cortisol, only 2 sponges were analyzed: the numbers in parentheses are the total number of vessels and endothelial cells of the 15 fields of the two sponges.

(c) Vessels and endothelial cells were identified by indirect immunoperoxidase staining with rabbit anti-vWF antibody.

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In other systems, native heparin and glucocorticosteroids have synergistic anti-angiogenic activity (Crum et al., 1985, Folkman, 1985). The inventor therefore determined the effect of injecting a mixture of native heparin and cortisol daily into implanted sponges on the vascularization of the sponge. The treatment neither retarded the ^{133}Xe clearance rate nor reduced the number of blood vessels in the sponge (Fig. 9a and Table VI).

A further study was performed in which mice were given daily intraperitoneal injections of high doses of heparin-cortisol (2.35mg) for 5 days and then half this dose (1.17mg) for 7 days. The dose was reduced during the study because the mice showed local bruising of the abdomen in the vicinity of the injection site. Measurements of ^{133}Xe clearance rates showed that vascularization was significantly ($P < 0.05$) retarded only while the larger doses were being given. Mice treated with equivalent amounts of a mixture of unconjugated heparin hydrazide and cortisol showed similar ^{133}Xe clearance rates to those in recipients of saline alone (Fig. 9b).

The above data indicates that the conjugate-mediated inhibition of DNA synthesis and cell migration observed with cultured vascular endothelial cells also occurs in vivo. Vascularization is a complex process requiring both the migration of vascular endothelial cells into the sponge and then proliferation to enable the capillary sprout to elongate. Injection of heparin-cortisol into implanted sponges resulted in the total abolition of vascularization, whereas the administration of unconjugated mixtures of heparin and cortisol, or heparin hydrazide and cortisol, did not affect vascularization. This last result is in contrast to other reports (Crum et al., 1985; Folkman, 1985).

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It should also be noted that neither heparin-adipic hydrazide nor heparin-adipic hydrazide-cortisol caused any loss of body weight or other outward signs of toxicity when administered intravenously to mice at doses of 10 mg per day for 14 days. By comparison, intravenous administration of cortisol itself at 0.4 mg per day killed three out of three mice within 7 days. Unmodified heparin was also toxic: two out of three mice which were injected intravenously with 0.5 mg heparin per day died within 3 days and the third died after 6 days.

EXAMPLE VI

ANTI-TUMOR EFFECTS OF HEPARIN-CORTISOL IN VIVO

1. Methods

Male C57 BL/6 mice aged 8-12 weeks were injected subcutaneously with 3×10^6 Lewis lung carcinoma (3LL) cells which had been maintained in tissue culture. The tumor is an anaplastic epidermoid carcinoma which originated as a spontaneous lung tumor in a C57BL mouse in 1951 (Sugiura & Stock, 1955). When the tumors had grown to measurable dimensions (about 0.3cm in diameter), the mice were injected intravenously with 0.1ml of saline containing 1mg heparin-cortisol every day for 9 days. Other mice received equivalent quantities of heparin-hydrazide (0.85mg), cortisol (0.15mg), or a mixture of both. On the tenth day, the mice were sacrificed and their tumors were removed and weighed. All treatment groups contained 8-10 mice. Differences in tumor weights were tested for statistical significance using Student's t-test and the Mann-Whitney-Wilcoxon (1976) Nonparametric Test for Two Independent Samples.

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2. Results and Discussion

In the first series of studies, daily intravenous injections of heparin-cortisol (1mg) into mice bearing
5 established subcutaneous Lewis lung carcinomas reduced the average weight of the tumors to 35% of that in mice treated with saline alone and to 60% of that in mice treated with an unconjugated mixture of heparin hydrazide (0.85mg) and cortisol (0.15mg) (Fig. 10). These
10 differences were statistically significant to the level of $P < 0.01$ and $P \leq 0.05$, respectively. In mice receiving cortisol alone (0.15mg/day), the average tumor weights were the same as in the group receiving both heparin hydrazide and cortisol. No reduction in tumor
15 weight was observed in mice receiving heparin hydrazide alone (0.85mg/day). Analogous results were obtained in two separate studies.

Next, in four separate studies, it was found that
20 daily intravenous injections of heparin-adipic hydrazide-cortisol (1 mg) into mice bearing established subcutaneous Lewis lung carcinomas reduced the average weight of the tumors to between 38% and 58% of that in mice treated with saline alone (Table VII). In all these
25 experiments, the retardation of tumor growth was highly statistically significant ($P < 0.001$). The antitumor effect of the conjugate was consistently superior to that of an equivalent dose of an unconjugated mixture of heparin-adipic hydrazide and cortisol which reduced tumor
30 weights to between 58% and 82% of that in mice treated with saline alone. In two of these experiments, the superiority of the conjugate over the mixture was statistically significant ($P < 0.05$) and in the third experiment it bordered on being significant ($P = 0.08$).
35 In mice receiving cortisol alone (0.15 mg/day), the average tumor weights were the same as in the group receiving both heparin-adipic hydrazide and cortisol. No

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reduction in tumor weight was observed in mice receiving
HAH alone (0.85 mg/day).

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TABLE VII
ANTI-TUMOR EFFECTS OF HEPARIN-ADIPIC HYDRAZIDE
(HAH)-CORTISOL IN MICE BEARING ESTABLISHED

5

SUBCUTANEOUS 3LL TUMORS

10

Experiment No.	Treatment ^(a)	Number of Mice	Tumor weight ^(b) (g)	T/C
1.	HAH-cortisol saline	10	0.78 ±	0.47
		10	0.47* ±	-
			1.66 ±	
			0.82	
2.	HAH-cortisol HAH+cortisol saline	10	0.64 ±	0.38
		10	0.39* ±	0.58
		10	0.97 ±	-
			0.43* ±	
3.	HAH-cortisol HAH+cortisol saline	6	1.68 ±	
		6	0.61	
		8	0.46 ±	0.44
			0.07*† ±	0.61
4.	HAH-cortisol HAH+cortisol HAH cortisol saline	8	0.63 ±	-
		8	0.30* ±	
		8	1.04 ±	
		8	0.30	
		10	1.11 ±	0.58
			0.24*† ±	0.82
			1.56 ±	1.16
			0.33	0.85
			2.21 ±	-
			0.47	
			1.62 ±	
			0.25	
			1.90 ±	
			0.54	

15 (a) Mice bearing 0.2-0.3 cm subcutaneous 3LL tumors were given daily i.v. injections of heparin-adipic hydrazide (HAH)-cortisol (1 mg) or equivalent amounts of unconjugated heparin-adipic hydrazide (0.83 mg), cortisol (0.17 mg) or a mixture of both.

20 (b) Tumor weight (± S.D) 7 days after onset of treatment.

* = tumor weight significantly ($P \leq 0.05$) less than in saline controls.

25 † = tumor weight in heparin-adipic hydrazide (HAH)-cortisol recipients significantly ($P \leq 0.05$) less than in recipients of heparin-adipic hydrazide (HAH) plus cortisol.

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In a further study, the treatment with heparin-cortisol was started three days before the 3LL cells were given and was continued for 10 days. The relative tumor weights were almost the same as those shown in Fig. 10. Hence, earlier treatment did not improve the anti-tumor effects of the heparin-cortisol.

The mechanism of action of heparin-cortisol is believed to be similar to that represented in Fig. 11. It is possible that the inhibitory effects of systemically administered heparin-cortisol on tumor growth and sponge vascularization might be improved by a different dose regimen designed to increase and sustain blood levels. Since the effects of steroids on cell activation and proliferation rapidly reverse when the steroid is removed (Folkman et al., 1973, 11690), it is possible that a wave of endothelial cell proliferation occurs towards the end of each 24 hour interval between injections. If so, the problem should be solved simply by a continuous infusion of the conjugate.

It is also possible that heparin-cortisol is rapidly removed by blood vessels in normal tissues following systemic administration, and that little drug reaches blood vessels in the tumor or sponge. However, this is highly unlikely. As the total number of vascular endothelial cells in the mouse is approximately 5×10^8 (Anggard, 1990) and the number of heparin receptors per endothelial cell is 10^6 to 10^7 (Bârzu et al., 1986), the entire vasculature of the mouse contains a total of 0.5 – 5×10^{15} heparin receptors. A standard dose of heparin-cortisol (1mg/injection) would supply approximately 5×10^{16} heparin-cortisol molecules, which exceeds the total number of heparin receptors by 10 to 100-fold. Thus, saturation of heparin receptors on blood vessels in the tumor or sponge is predicted to occur after each

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administration of heparin-cortisol. Biodistribution studies are needed to confirm this prediction.

Despite compelling evidence to the contrary
5 (Folkman, 1990), it is possible that solid tumors are not completely angiogenesis-dependent and that tumor growth can continue, even in the presence of an angiogenesis inhibitor, by infiltration of tumor cells along existing vascular tracts. This appears to be true of lymphomas
10 (Denekamp & Hobson, 1982) and it is possible that the Lewis lung carcinoma used in the present study resembles lymphomas in this regard. Measuring the rate of endothelial cell division in tumors in mice on heparin-cortisol therapy will allow this point to be resolved.

15

The inventor proposes that angiostatic steroids (e.g. tetrahydro S) lacking glucocorticoid and mineralocorticoid activity (Crum et al., 1985) may be substituted for cortisol in the conjugate. These
20 steroids appear to be ideal for coupling to heparin because they should give potent anti-angiogenic in animals without causing the toxic side effects normally associated with long term corticosteroid therapy. Long term treatment is likely to be needed in tumor therapy to
25 suppress the outgrowth of dormant or anoxia-resistant tumor cells.

In conclusion therefore, the major findings to emerge in the present study are that heparin-cortisol
30 inhibits DNA synthesis and migration of vascular endothelial cells *in vitro*, retards vascularization of sponges implanted into mice, and significantly inhibits the growth of solid Lewis lung carcinomas in mice. In each assay, the activity of the heparin-cortisol
35 conjugate exceeded that of a mixture of its components showing that chemical linkage of the components improves the biological activity of the conjugate. The heparin-

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cortisol conjugate of the present invention thus represents the prototype of a new class of angiogenesis inhibitors with anti-tumor activity. By refining the choice of steroid and carrier moieties and the dose regimen for the administration of the conjugates the inventor proposes that the clinical usefulness of these drugs will be increased.

10

* * *

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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by reference to the extent that they supplement, explain,
provide a background for or teach methodology, techniques
and/or compositions employed herein.

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CLAIMS

1. A conjugate comprising a polyanionic compound
5 conjugated to a selected pharmacological agent.
2. The conjugate of claim 1, wherein the polyanionic
compound comprises a polysulfated or polysulfonated
10 compound.
3. The conjugate of claim 1, wherein the polyanionic
compound comprises a polyanionic polymer.
15
4. The conjugate of claim 3, wherein the polyanionic
polymer comprises a polysulfated or polysulfonated
polymer.
20
5. The conjugate of claim 4, wherein the polysulfated
polymer is heparin or a heparin derivative.
25
6. The conjugate of claim 5, wherein the heparin
derivative is a non-anticoagulating heparin derivative.
- 30 7. The conjugate of claim 6, wherein the conjugate has
less than 2.5% of the anticoagulating activity of native
heparin.
- 35 8. The conjugate of claim 2, wherein the polysulfated
compound is suramin or a suramin derivative.

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9. The conjugate of claim 4, wherein the polysulphated polymer is heparan sulfate, chondroitin sulfate, keratan sulfate, dermatan sulfate, sulfated chitin, sulfated chitosan, sulfated alginic acid, pentosan polysulfate, a
5 sulfated cyclodextrin, polystyrene sulfonate, sulfated polyvinyl alcohol, polyvinyl sulfate or polyethylene sulfonate.

10 10. The conjugate of claim 1, wherein the polyanionic compound is derivatized to introduce functional groups permitting the attachment of a selected agent through a biologically releasable bond, and the selected agent is
15 conjugated to the polymer by means of the biologically releasable bond.

11. The conjugate of claim 10, wherein the polyanionic compound is derivatized to introduce functional groups
20 permitting the attachment of a selected agent through a disulfide or trisulfide bond, and the selected agent is conjugated to the polymer through the introduced functional groups.

25

12. The conjugate of claim 11, wherein the introduced bond is a selectively hydrolyzable bond.

30 13. The conjugate of claim 12, wherein the selectively hydrolyzable bond is an acid-labile bond.

14. The conjugate of claim 13, wherein the acid-labile
35 bond is hydrolyzable at a pH of 4.8 or lower.

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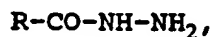
15. The conjugate of claim 13, wherein the polyanionic compound is derivatized to introduce side chains terminating in hydrazide, hydrazine, primary amine or secondary amine groups.

5

16. The conjugate of claim 15, wherein the derivatized polyanionic compound is the condensation product of the compound and adipic dihydrazide, succinic dihydrazide, hydrazine or hydrazine hydrate, and the agent is conjugated to the compound through a Schiff's base linkage.

17. The conjugate of claim 16, wherein the agent is conjugated to the compound through a hydrazone or acyl hydrazone bond.

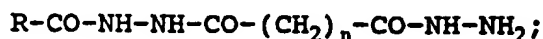
18. The conjugate of claim 17, wherein the agent is conjugated to the compound through a hydrazide linker having the structure:



25

wherein R = the polyanionic compound.

19. The conjugate of claim 16, wherein the agent is conjugated to the compound by a hydrazide linker having the structure:



35

wherein n = 1-50; and

R = the polyanionic compound.

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20. The conjugate of claim 10, wherein the biologically releasable bond is an enzyme-sensitive bond.

5 21. The conjugate of claim 20, wherein the enzyme-sensitive bond is a peptide bond.

10 22. The conjugate of claim 21, wherein the agent is linked to the compound through a peptide that includes the sequence Leu-Ala-Leu-Ala.

15 23. The conjugate of claim 20, wherein the enzyme-sensitive bond is an ester, an amide, a phosphodiester or a glycoside.

20 24. The conjugate of claim 1, wherein the selected agent is an agent from Table I or Table III.

25 25. The conjugate of claim 1, wherein the selected agent is biotin, a phorbol ester, adenosine, inosine, hypoxanthine, nicotinamide, prostaglandin E2 or a calcium ionophore.

30 26. The conjugate of claim 1, wherein the selected agent is ricin A-chain.

27. The conjugate of claim 1, wherein the selected agent is a steroid.

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28. The conjugate of claim 27, wherein the steroid is a steroid from Table II or Table IV.

5 29. The conjugate of claim 27, wherein the steroid is a synthetic steroid.

30. The steroid conjugate of claim 27, wherein the
10 steroid has anti-angiogenic activity.

31. The conjugate of claim 27, wherein the steroid
employed for conjugating to the compound has one or more
15 free ketone or aldehyde groups.

32. A method for preparing a conjugate, comprising:

20 condensing in a reaction mixture a polyanionic
 compound with a derivatizing agent to form a
 derivatized polyanionic compound;

 removing from the reaction mixture any unreacted
25 material from said derivatized polyanionic
 compound;

 adding to the reaction mixture the selected agent to
 be conjugated; and

30 incubating said reaction mixture to create a
 polyanionic compound-agent conjugate.

35 33. The method of claim 32, wherein the selected agent
 comprises an agent from Table I or III, a steroid, a

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phorbol ester, adenosine, inosine, hypoxanthine,
nicotinamide, prostaglandin E2 or a calcium ionophore.

- 5 34. The method of claim 32, wherein the polyanionic
compound is heparin or suramin.
- 10 35. The method of claim 32, further defined as a method
for preparing a heparin-steroid conjugate comprising the
steps of:
- 15 condensing heparin with hydrazine or a hydrazide
 derivative, using an excess of water soluble
 carbodiimide at an acidic pH, to form a
 heparin-derivative having one or more side
 chains terminating in hydrazide groups;
- 20 removing the unreacted material from said heparin-
 hydrazide derivative;
- adding a steroid solution at acidic pH to form a
 reaction mixture;
- 25 incubating said reaction mixture; and
- adjusting the pH of said heparin-steroid conjugate
 to pH 7 or above.
- 30 36. A pharmaceutical preparation of a conjugate
comprising the conjugate of claim 1 dispersed in a
pharmacologically acceptable vehicle.
- 35 37. The pharmaceutical preparation of claim 36, suitable
for parenteral administration.

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38. A method for targeting an agent to a vascular endothelial cell, comprising:

preparing a conjugate in accordance with claim 1;

5 and

exposing vascular endothelial cells to said conjugate.

10

39. A method for inhibiting DNA synthesis, cellular proliferation or cellular migration in vascular endothelial cells, comprising:

15 preparing a conjugate in accordance with claim 30;
and

exposing vascular endothelial cells to said conjugate.

20

40. A method for stimulating DNA synthesis and cellular proliferation in vascular endothelial cells, comprising:

25 preparing an endothelial cell-targeted conjugate in accordance with claim 25; and

exposing vascular endothelial cells to said conjugate.

30

41. The use of a conjugate comprising a polyanionic compound conjugated to a selected pharmacological agent in the preparation of a medicament for affecting
35 angiogenesis.

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42. A use according to claim 41, wherein the selected agent comprises a steroid having anti-angiogenic activity and the medicament is intended for use in inhibiting angiogenesis.

5

43. A use according to claim 42, wherein the selected agent comprises a steroid having anti-angiostatic activity, and wherein the medicament is intended for use in the treatment of cancer.

10

44. A use according to claim 41, wherein the selected agent comprises biotin, a phorbol ester, adenosine, inosine, hypoxanthine, nicotinamide, prostaglandin E2, or a calcium ionophore, and wherein the medicament is intended for use in stimulating angiogenesis.

15

45. A use according to claim 41, wherein the polyanionic compound is herparin or suramin.

20

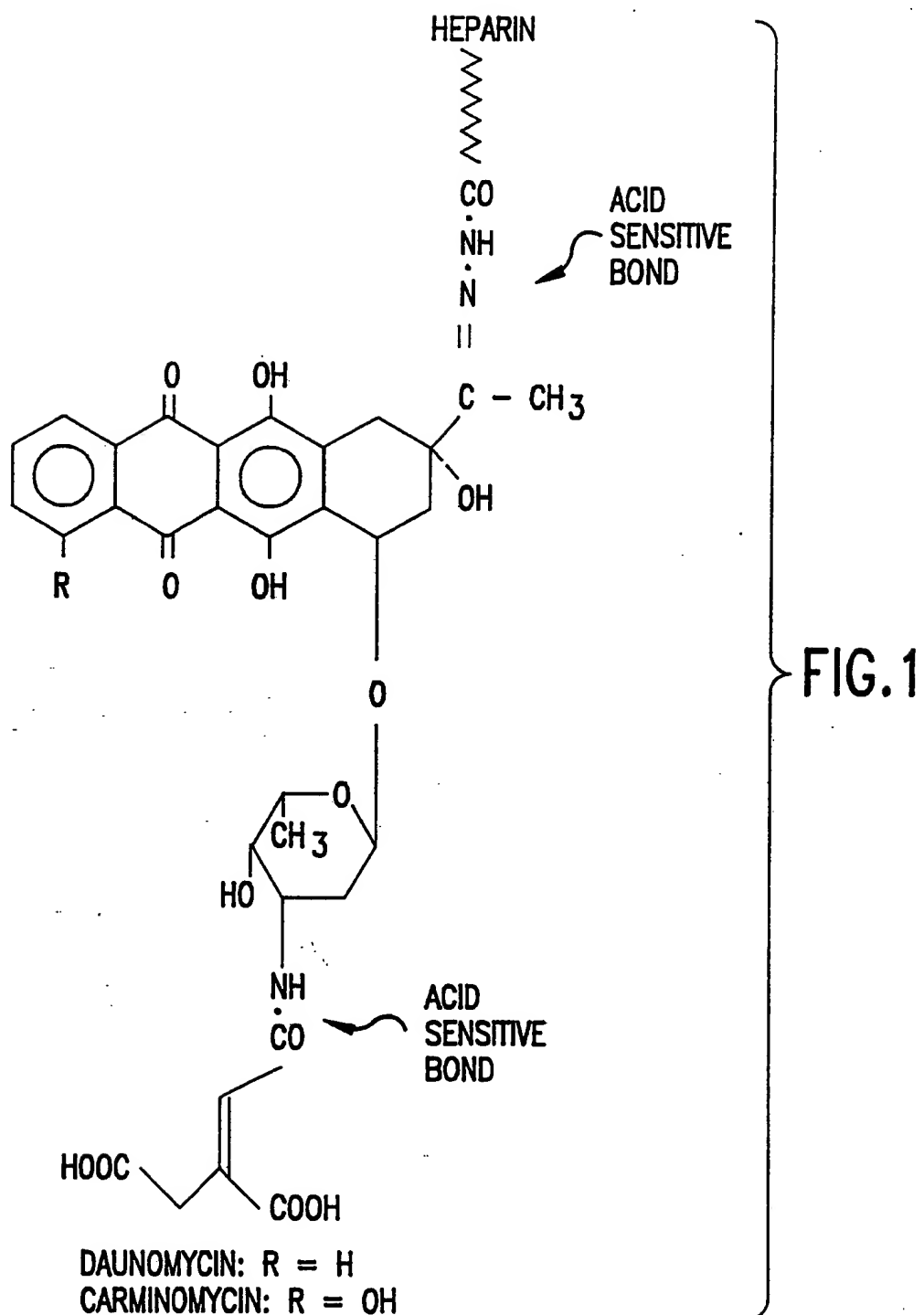
46. A use according to claim 41, wherein the medicament is suitable for parenteral administration.

25

47. A use according to claim 41, wherein the medicament is encapsulated within a biodegradable polymer or slow-release capsule.

30

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SUBSTITUTE SHEET

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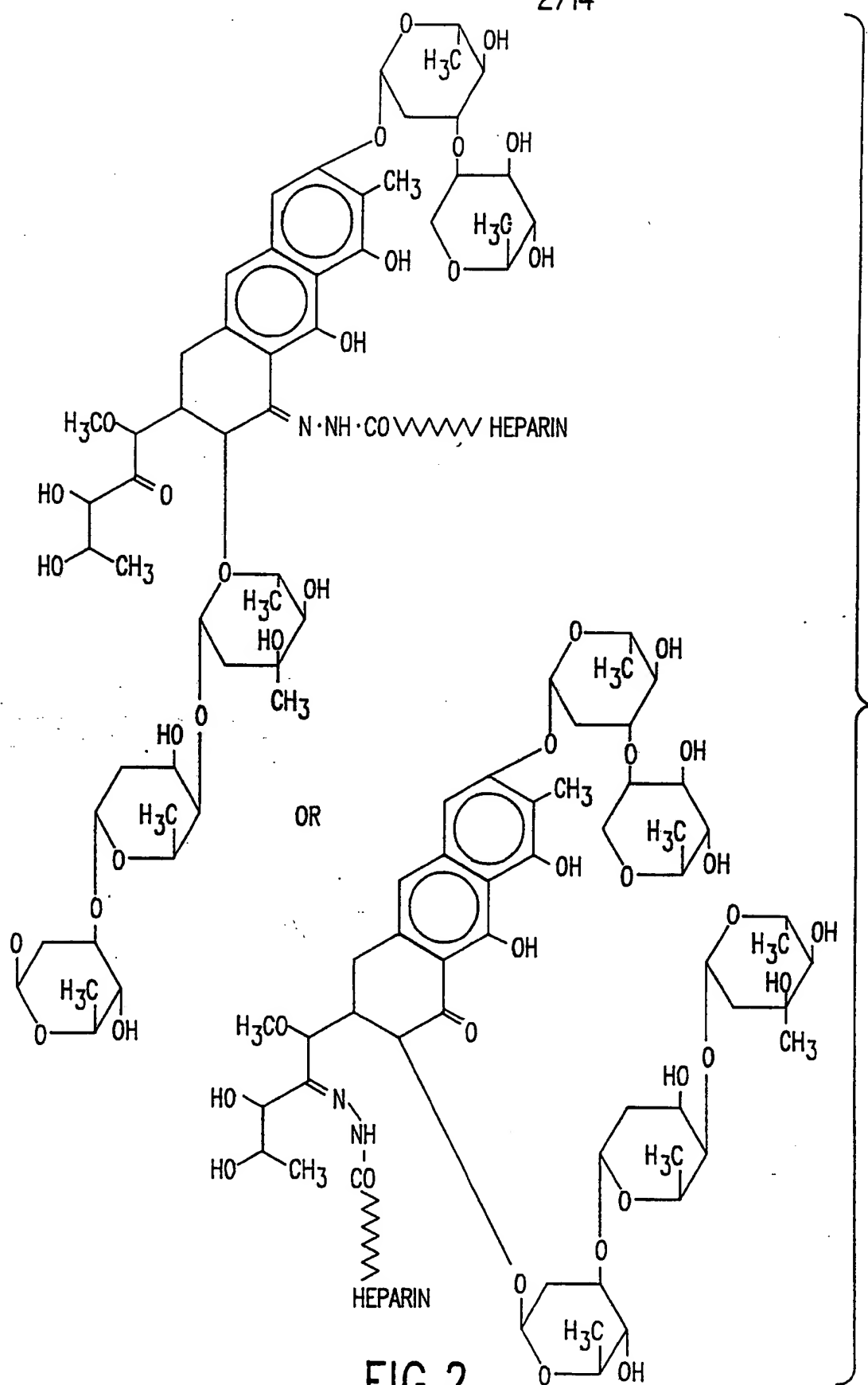


FIG.2
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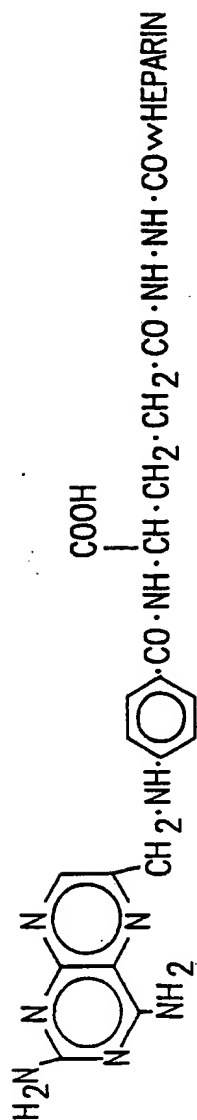


FIG. 3

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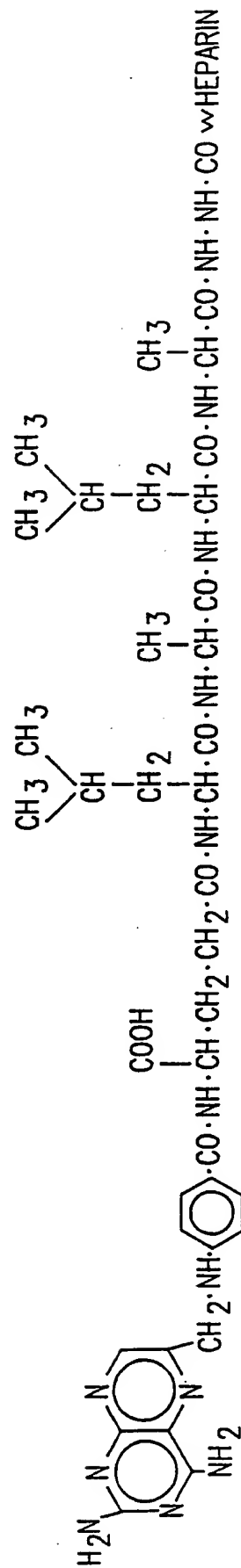


FIG. 4

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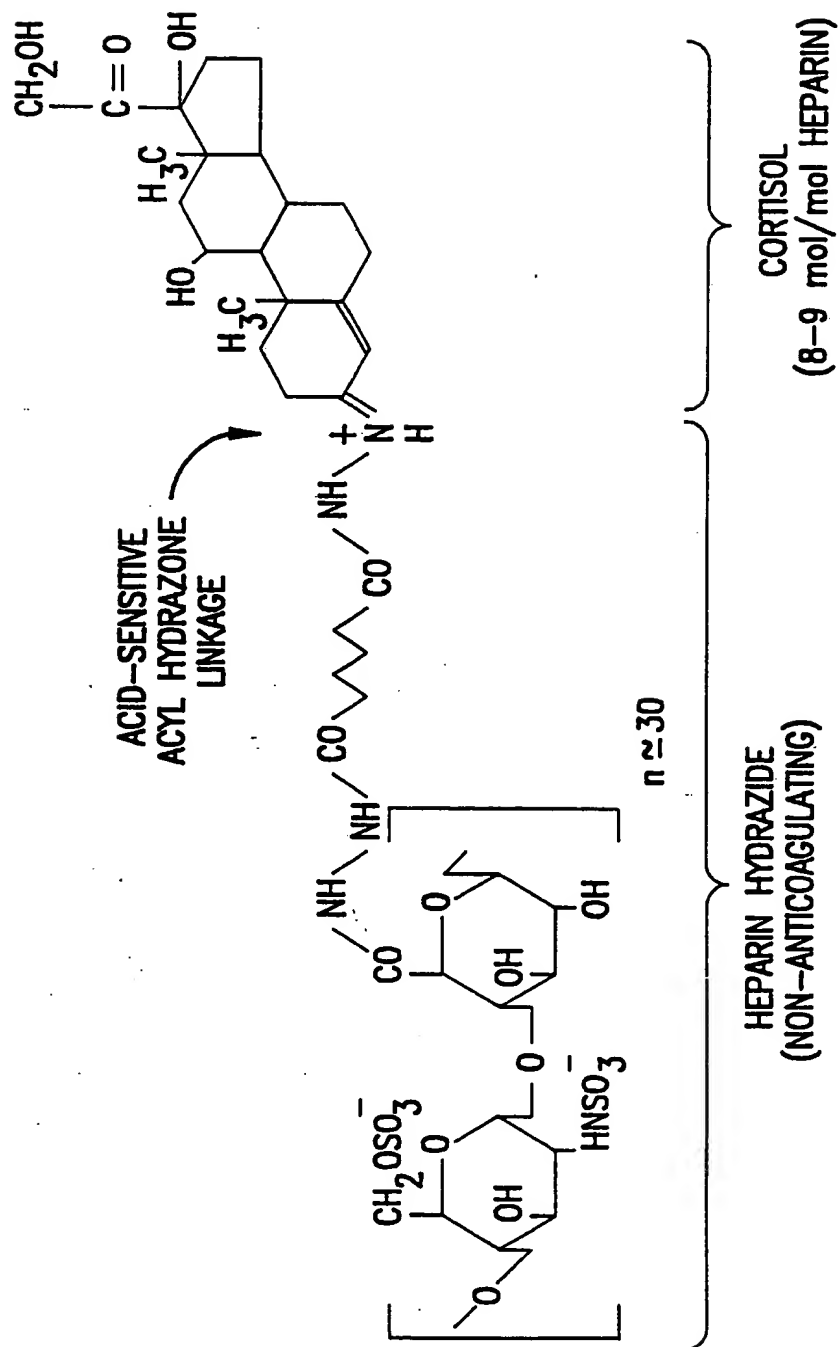


FIG.5

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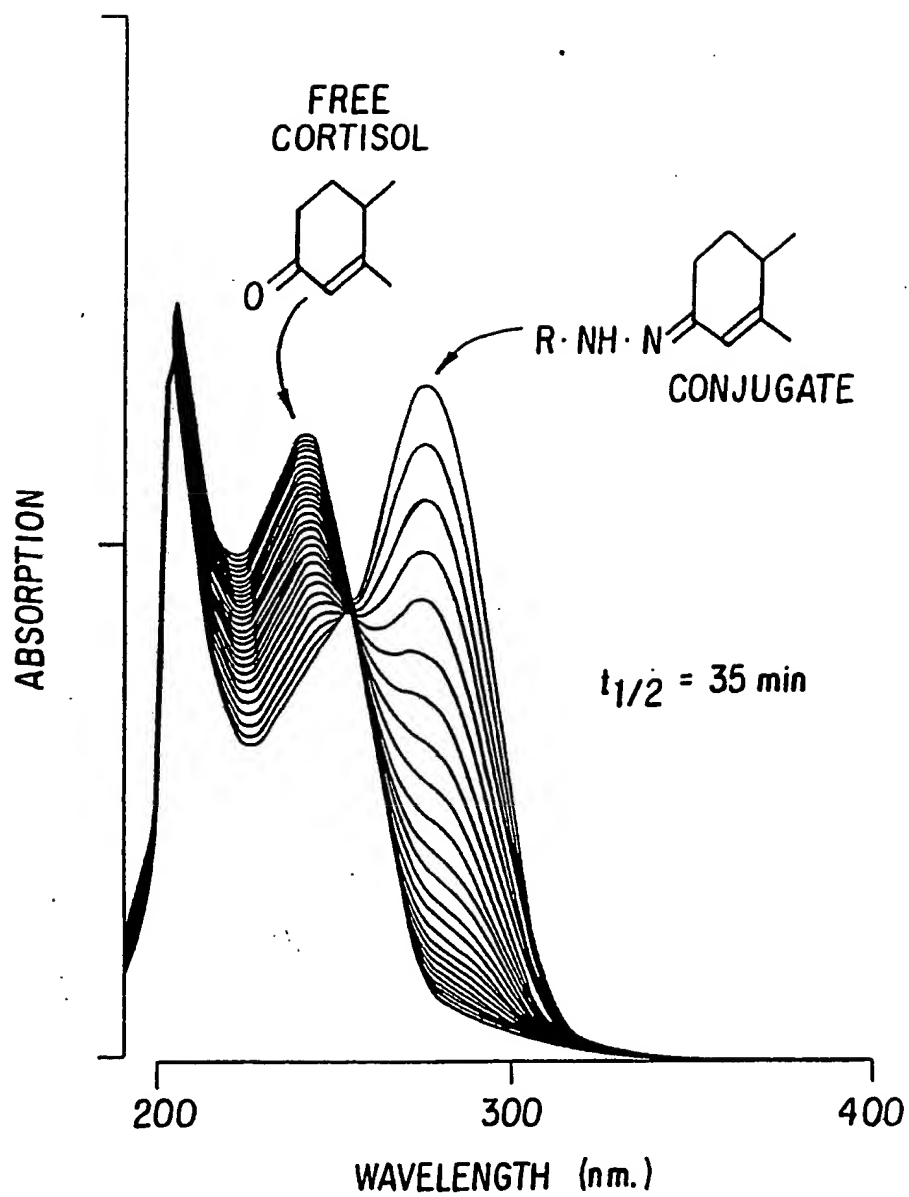


FIG. 6

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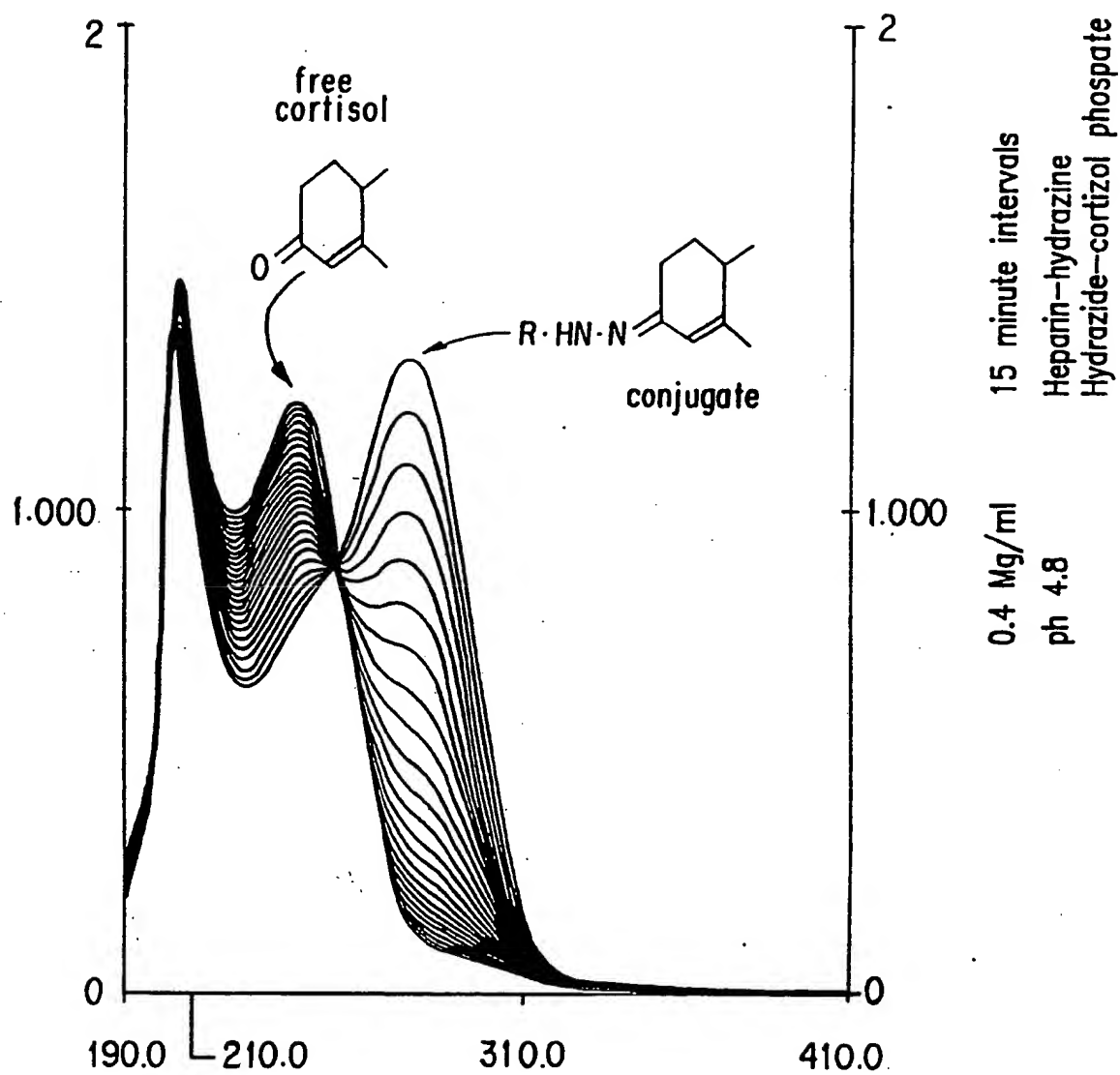
 $t_{1/2} = \text{at pH } 4.8 = 1.5 \text{ hours}$

FIG. 7A

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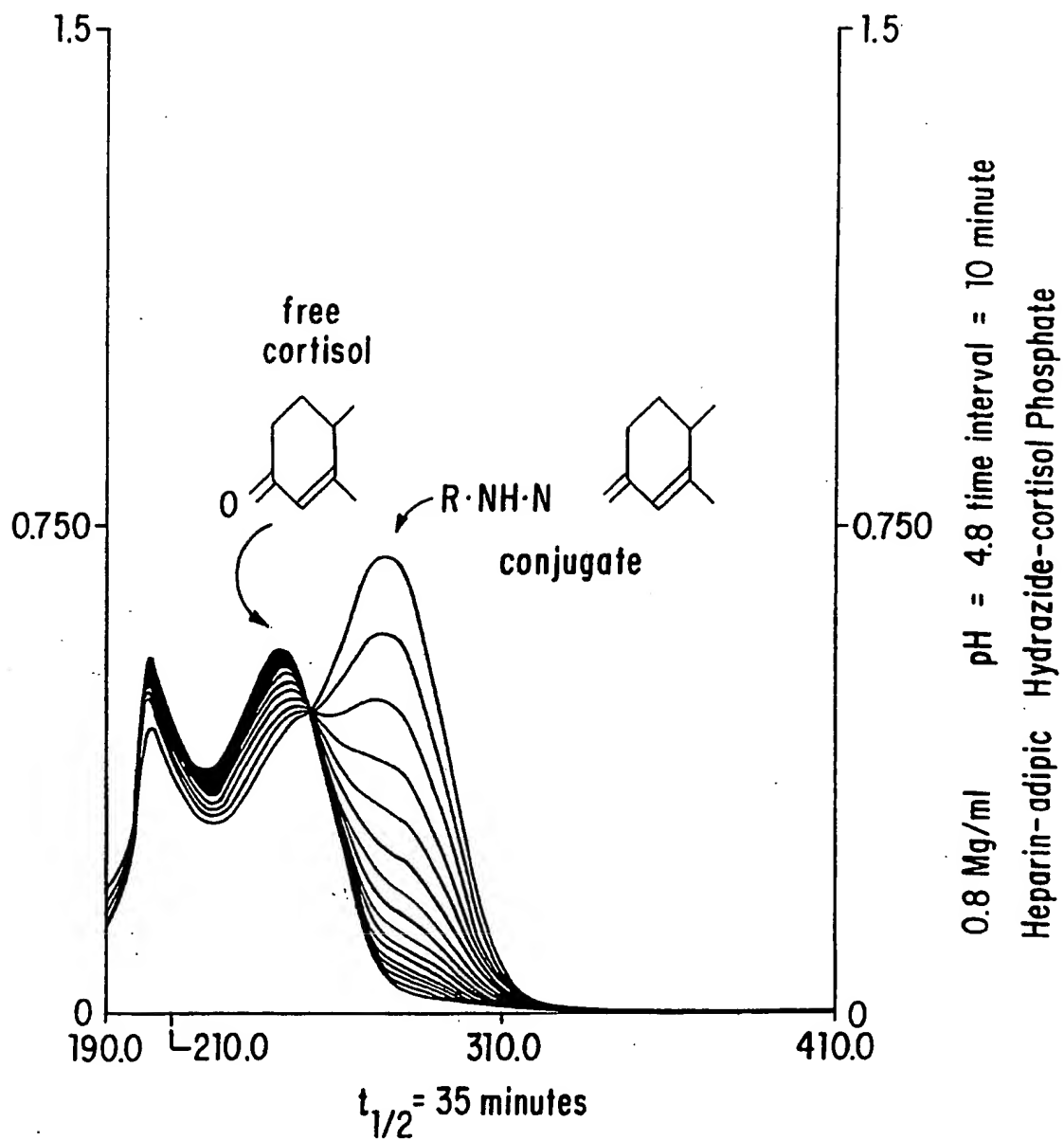


FIG. 7B

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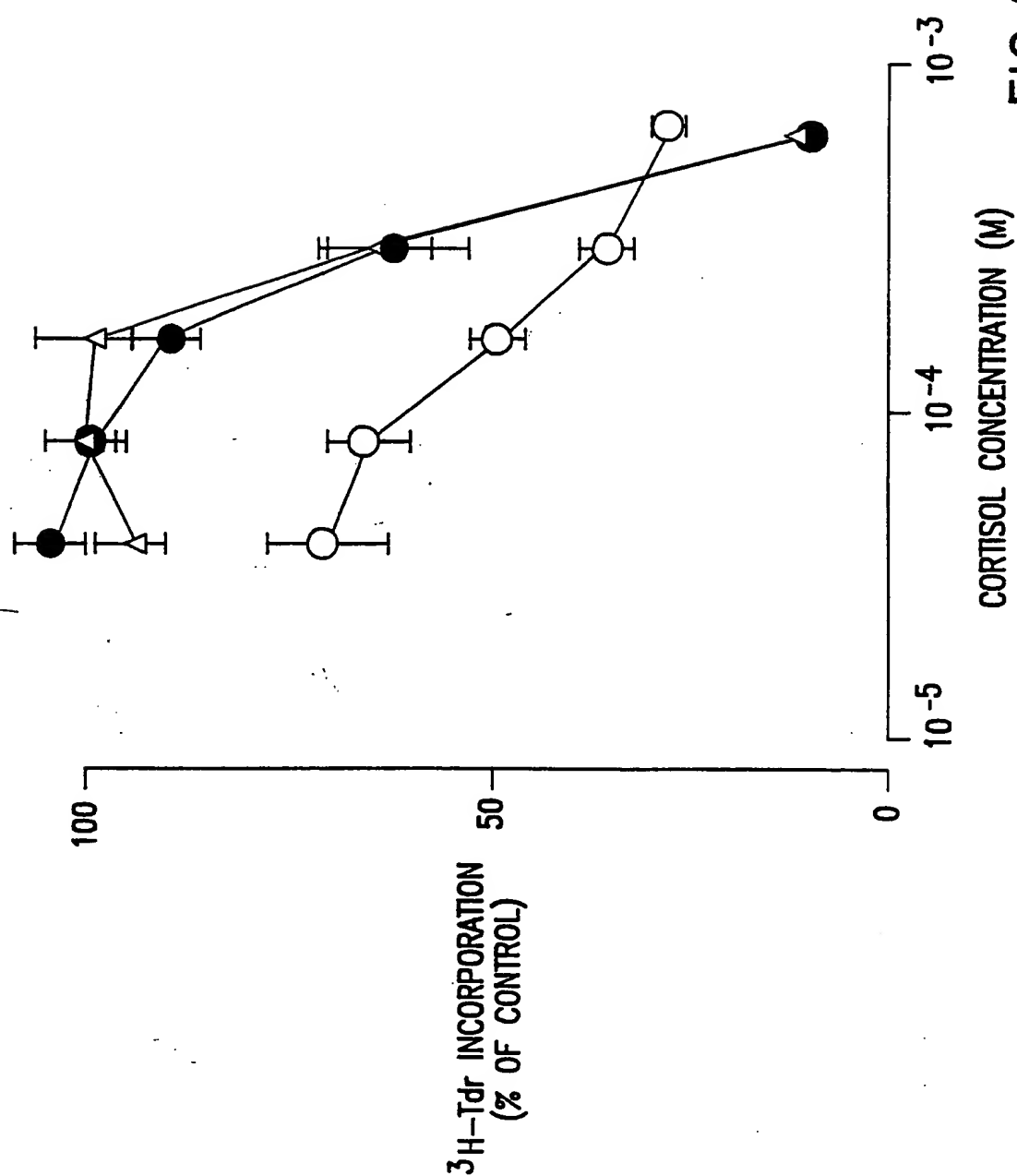
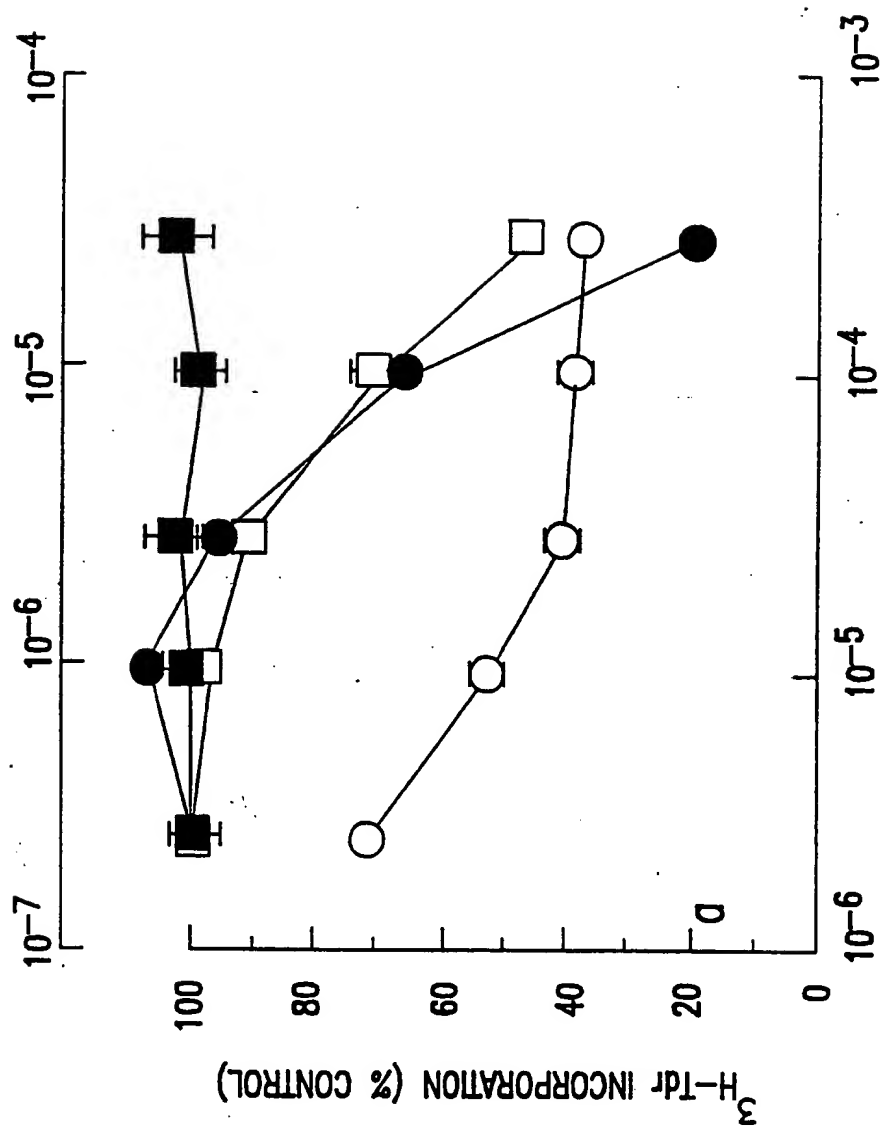


FIG. 8A

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FIG.8B
CORTISOL CONCENTRATION (M)

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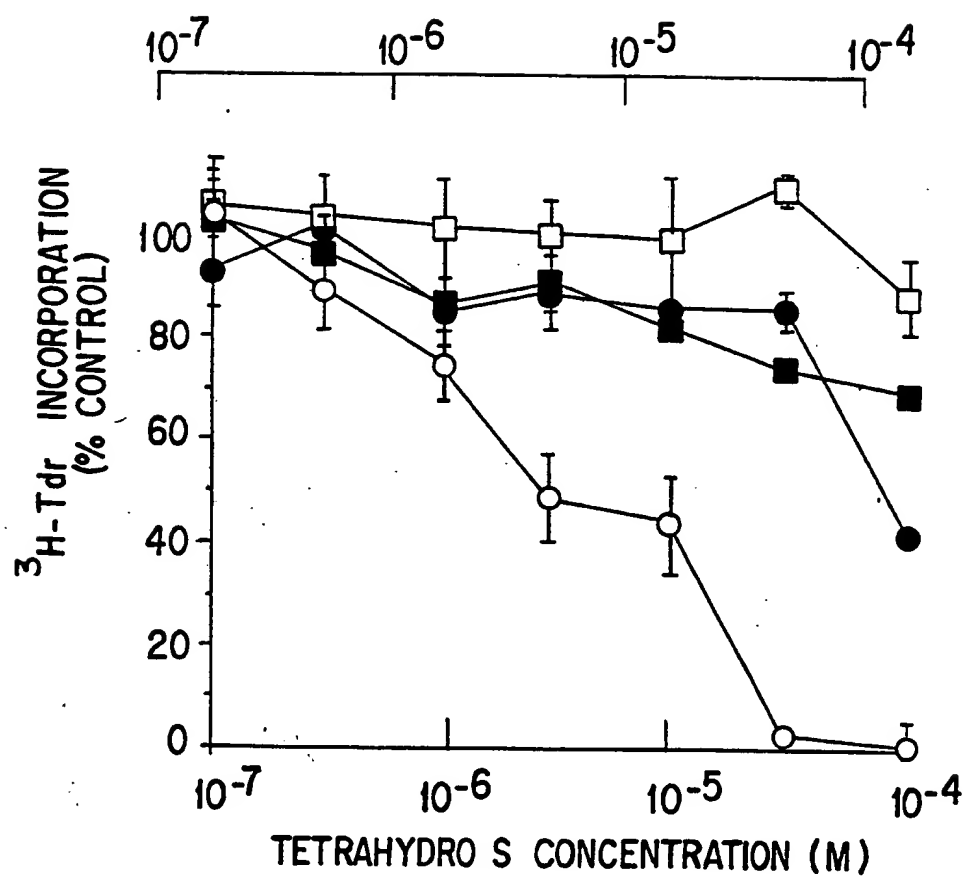


FIG. 8C

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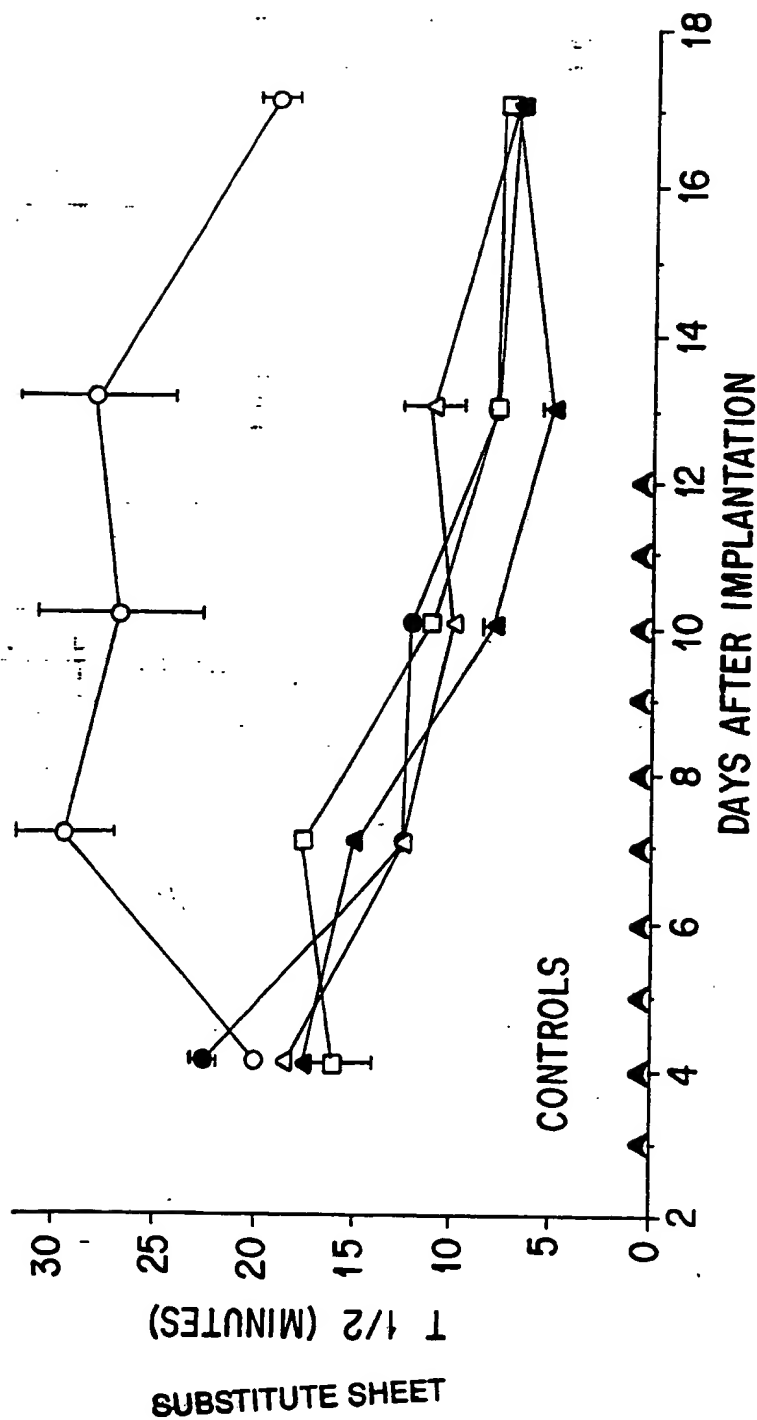


FIG.9A

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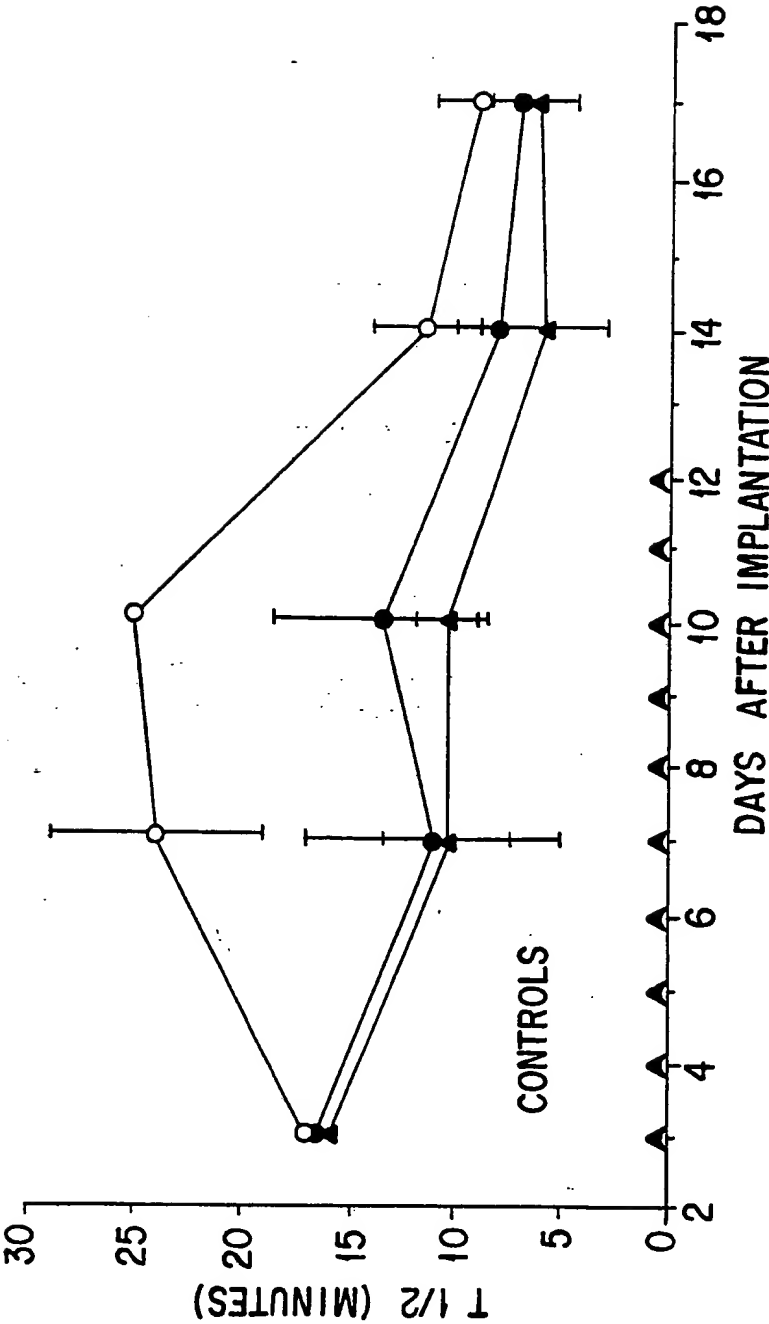


FIG.9B

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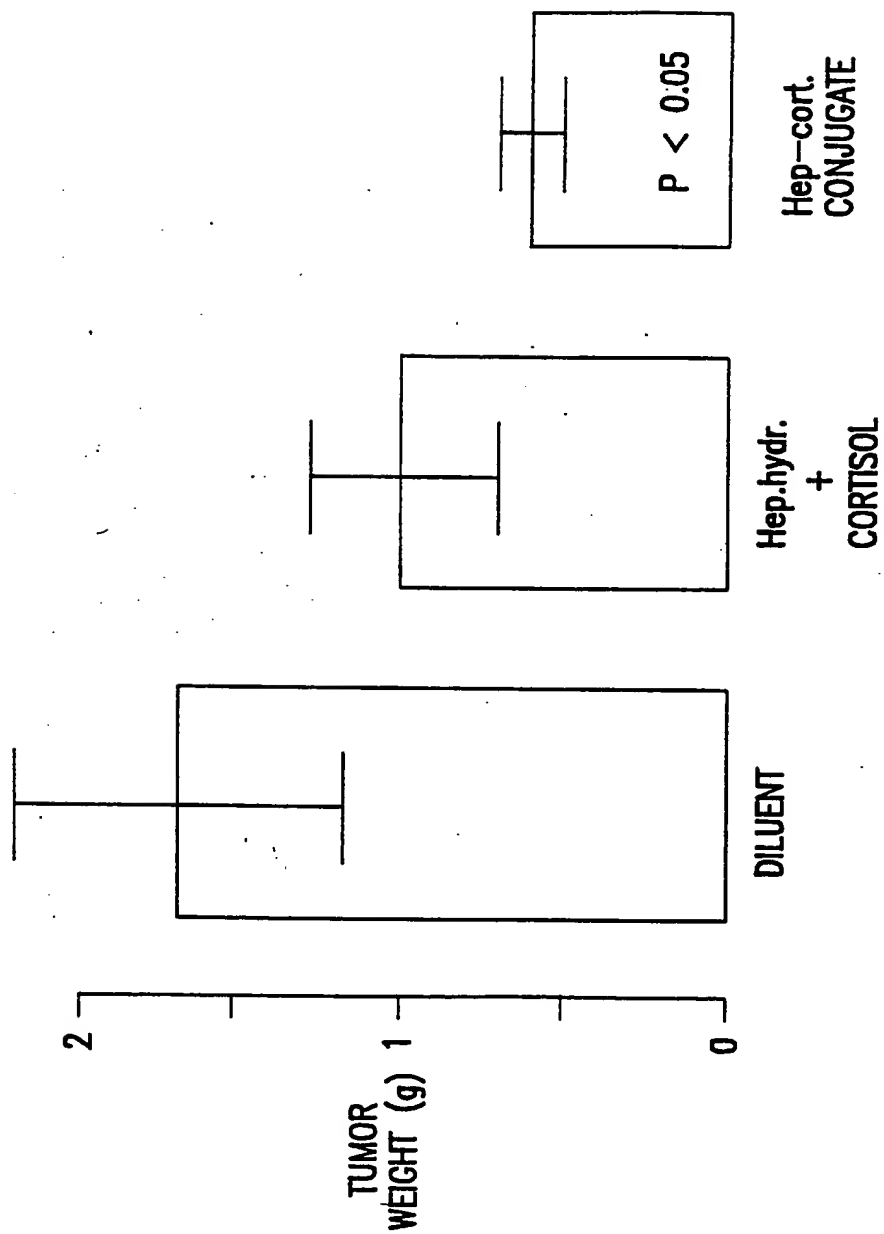
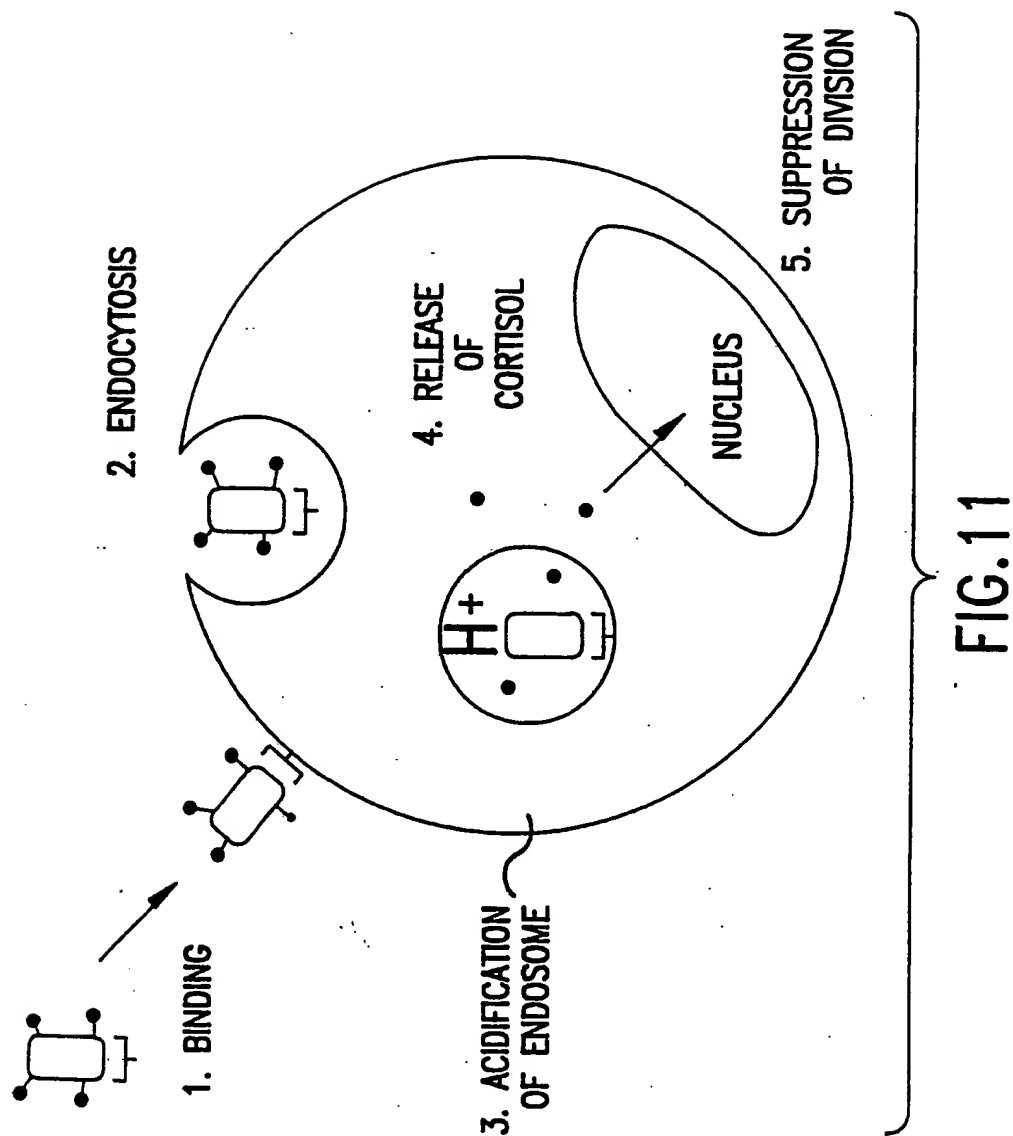


FIG.10

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/02619

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A61K47/48		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	INVEST. OPHTHALMOL. VISUAL SCI. vol. 29, 1988, page 403 A. FENSELAU ET AL. 'HEPARIN CONJUGATES: NOVEL ANTIANGIOGENIC AGENTS FOR POSSIBLE USE IN RETINOPATHY TREATMENT'	1-6, 10, 12-13, 20, 27-31, 36-39, 41-43, 45-46 8-9, 40
Y	see the whole document	
Y	SCIENCE vol. 243, 1989, pages 1490 - 1493 J. FOLKMAN ET AL. 'CONTROL OF ANGIOGENESIS WITH SYNTHETIC HEPARIN SUBSTITUTES' cited in the application	9, 40
A	see the whole document	1-8, 27-31, 36-47
--- -/-		
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
08 JULY 1993	22. 07. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	HOFF P.J.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,9 015 816 (THE UPJOHN COMPANY) 27 December 1990	8
A	see abstract; claims	1-7, 27-31, 36-47
X	--- JOURNAL OF PHARMACEUTICAL SCIENCES vol. 75, no. 2, 1986, pages 172 - 175 H. JACOBS ET AL. 'IN VITRO BIOACTIVITY OF A SYNTHESIZED PROSTAGLANDIN E1-HEPARIN CONJUGATE' see the whole document	1-5, 24-25, 36-37
X	--- WO,A,8 300 150 (WALTON A.) 20 January 1983	1-4, 9-10,12, 15,20, 23-24, 32-33, 36-37
A	see abstract see page 4, line 9 - page 6, line 35; claims	16-18
X	--- 'CONTROLLED RELEASE DELIVERY SYSTEMS' 1983 , MARCEL DEKKER, INC. , NEW YORK	1-5, 10-12, 17,20, 23, 32-34, 36-37
X	--- "CONTROLLED RELEASE FROM GLYCOSAMINOGLYCAN DRUG COMPLEXES" see page 107 - page 119	
X	--- JOURNAL OF BIOLOGICAL CHEMISTRY vol. 264, no. 14, 1989, pages 7950 - 7956 R. G. LEBARON ET AL. 'BINDING OF HEPARAN SULFATE TO TYPE V COLLAGEN' see page 7951	1-5,9,25
A	--- LIFE SCIENCE vol. 18, no. 9, 1976, pages 977 - 981 G.W. PASTERNAK ET AL. 'MACROMOLECULAR NALOXONE: A NOVEL LONG-ACTING POLYMER-BOUND DRUG' see the whole document	18

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	SCIENCE vol. 221, 1983, pages 719 - 725 J. FOLKMAN ET AL. 'ANGIOGENESIS INHIBITION AND TUMOR REGRESSION CAUSED BY HEPARIN OR A HEPARIN FRAGMENT IN THE PRESENCE OF CORTISONE' cited in the application see the whole document ---	1-47
A	THE CANCER JOURNAL vol. 2, no. 1, 1988, pages 9 - 13 N. SAKAMOTO ET AL. 'MECHANISM OF THE SYNERGISTIC EFFECT OF HEPARIN AND CORTISONE AGAINST ANGIOGENESIS AND TUMOR GROWTH' cited in the application see the whole document ---	1-47
A	CARBOHYDRATE RESEARCH vol. 16, 1971, pages 199 - 205 I. DANISHEFSKY ET AL. 'CONVERSION OF CARBOXYL GROUPS OF MUCOPOLYSACCHARIDES INTO AMIDES OF AMINO ACID ESTERS' cited in the application see the whole document -----	1-47

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/02619

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
In view of the large number of compounds which are defined by the terms "polyanionic compound" or "polysulfonated compound" in claims 1-4, the search was limited to the general idea of the invention, i.e., for the compounds mentioned in the claims 5-9 (PCT: Art. 6 Guidelines Part B, chapt. III, 3.7).
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9302619
SA 71730

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

08/07/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9015816	27-12-90	AU-A- 5640390	08-01-91
		EP-A- 0477195	01-04-92
		JP-T- 4506066	22-10-92

WO-A-8300150	20-01-83	AU-A- 8762982	02-02-83
		CA-A- 1188988	18-06-85
		EP-A- 0083367	13-07-83
		US-A- 4489065	18-12-84

EPO FORM P007

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82